

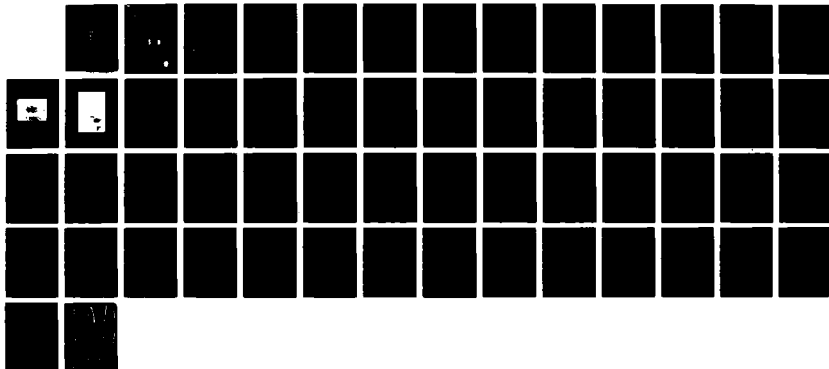
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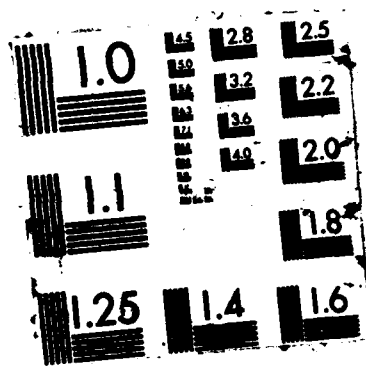
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**EFFECTS OF SIMULTANEOUS RADIOFREQUENCY
RADIATION AND CHEMICAL EXPOSURE OF
MAMMALIAN CELLS**

Volume I

Martin L. Meltz, Ph.D.

**University of Texas Health Science Center
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San Antonio, TX 78284**

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**Prepared for
USAF SCHOOL OF AEROSPACE MEDICINE
Human Systems Division (AFSC)
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NOTICES

This annual report was submitted by the Department of Radiology, the University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas, under contract F33615-84-C-0604, job order 2312-V7-18, with the USAF School of Aerospace Medicine, Human Systems Division, AFSC, Brooks Air Force Base, Texas. Dr. David N. Erwin (USAFSAM/RZP) was the Laboratory Project Scientist-in-Charge.

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

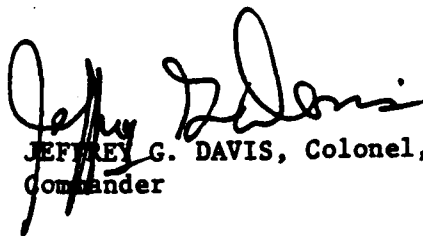
This report has been reviewed and is approved for publication.



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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The major objective of this project has been to determine whether radiofrequency radiation (RFR), at power densities and specific absorption rate (SAR) values which can result in temperature increases in the exposure medium, can affect the extent of chemically induced toxicity, mutagenicity, sister chromatid exchange, or chromosome aberrations in mammalian cells. The in vitro system which has been used for toxicity and mutagenicity studies (beginning in the first year of this project) is the mouse leukemic L5178Y cell thymidine kinase locus (TK +/-) mutation assay. The studies involving sister chromatid exchange and chromosome aberration induction will be performed in the second year in Chinese hamster ovary (CHO) cells, and subsequently in freshly isolated human lymphocytes. The chemicals employed in the L5178Y studies were mitomycin C (inducer of DNA interstrand cross links) and Adriamycin (inducer of DNA single-strand breaks). Ethyl methanesulfonate was used to verify the performance of the L5178Y mutation assay. The effective concentration ranges of Adriamycin and mitomycin C for a 4-hr treatment time at 37°C were established.					
(Cont'd. on p. ii)					
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19. ABSTRACT (Cont'd.)

The range determination allows selection of the chemical concentration which causes toxicity (measured by growth inhibition), but still permits enough growth and viability for successful mutagenicity studies.

In this first year of research, a major additional project was undertaken. This was the design and construction of an appropriate rotating water bath for exposure of tissue culture flasks. A series of preliminary RFR measurement experiments were completed, using the water baths. These included determinations of: (a) average power densities at different positions in the RFR field for 5.6 GHz pulse wave (PW), and 2.45 GHz continuous wave (CW) exposures; (b) SARs for 5.6 GHz and 2.45 GHz RFR exposures; and (c) temperature vs. time heating patterns in the flasks immersed in the RFR exposure water bath, in the exposure water bath itself, in the circulating water bath located outside of the anechoic chamber, and in the air in the anechoic chamber. This phase of the research was performed over the power range of 50- to 600-W forward power at 5.6 GHz (PW), and at 100- to 500-W forward power at 2.45 GHz (CW).

The biological studies performed focused on characterization of the L5178Y cell growth in suspension, and on the cloning efficiency of the cells in soft agar. The former included a comparison of growing the cells at 37°C with rotation vs. keeping them stationary; (b) using different pH buffering systems; and (c) after incubation for 4 hr at temperatures ranging from 37°C to 45°C. The latter included a comparison of the ability of the cells to form colonies in soft agar after: (aa) using different autoclaves to sterilize the agar; (bb) autoclaving the agar for different times; (cc) using different types of agar; and (dd) remelting the agar in an autoclave vs. in a boiling water bath prior to dilution of the agar.

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EFFECTS OF SIMULTANEOUS RADIOFREQUENCY RADIATION AND CHEMICAL EXPOSURE OF MAMMALIAN CELLS: VOLUME I

I. DESIGN AND CONSTRUCTION OF PLEXIGLAS WATER-BATH EXPOSURE CHAMBERS

In the experiments undertaken in this project, three factors had to be monitored and/or controlled:

The first and most obvious factor was the temperature increase which was expected to occur in the tissue culture medium in the T-25 exposure flasks when these were subjected to radiofrequency radiation (RFR) at higher power densities.

The second factor was the possible non-uniformity at different positions in the radiofrequency (RF) field.

The third factor was the possible creation of a chemical gradient in the microenvironment of individual cells, if they were allowed to remain stationary when they were exposed simultaneously to chemical and RF radiation.

To alleviate these factors, a special circulating water bath was designed. One of the three water baths was used for exposure of cells in flasks in the RFR field; one was used for performing a simultaneous temperature control exposure (i.e., a similar temperature increase vs. time pattern, produced by non-RFR convection heating); and one was used for incubating cells at 37°C. The latter two baths were placed in the anechoic chamber, but positioned outside of the RFR field.

The exposure water bath is shown in Fig. 1. Ten T-25 flasks are placed into "impressions" on the underside of a Styrofoam wheel; the wheel floats freely on the surface of the water about a central axle. This rotation provides both averaging of RF field inhomogeneities and a slight agitation of the culture medium to prevent the formation of chemical gradients, thus alleviating two of the concerns indicated above.

The water bath (Fig. 2), which measures 45.72 x 45.72 x 10.16 cm, is constructed of Plexiglas[®]. Four independently adjustable leveling feet, machined from Nylon[®], are attached to the base. A water inlet and an outlet port are set into two opposing sides of the water bath. The inlets, attached at a 45° angle, are constricted to generate a water jet. The jets are directed against the side of the wheel and propel it. The outlets are 1/2 in. lower than the inlets. A hollow cylindrical axle, which protrudes through the floor of the bath, provides access to one flask with a Vitek probe while the wheel is rotating. This allows continuous temperature monitoring during the RFR exposure. With the water circulators currently in use, rotational speeds of up to 20 rpm can be realized.

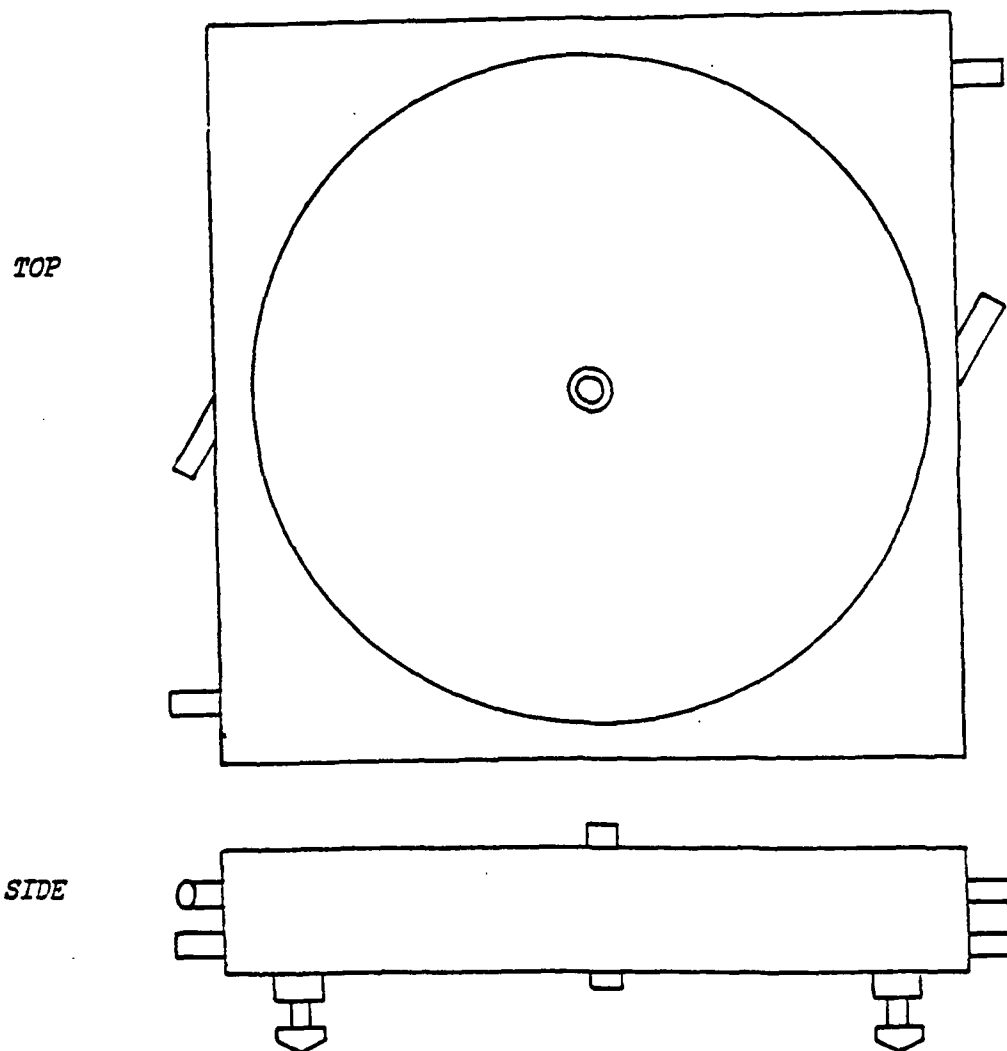


Figure 1. Overhead and side views of circulating exposure water bath.



Figure 2. Downward view of exposure water bath and top of circular flask support.

For RFR exposure, one of the water baths is placed on a Styrofoam[®] table and is centered under the horn (Fig. 3) in the far field.

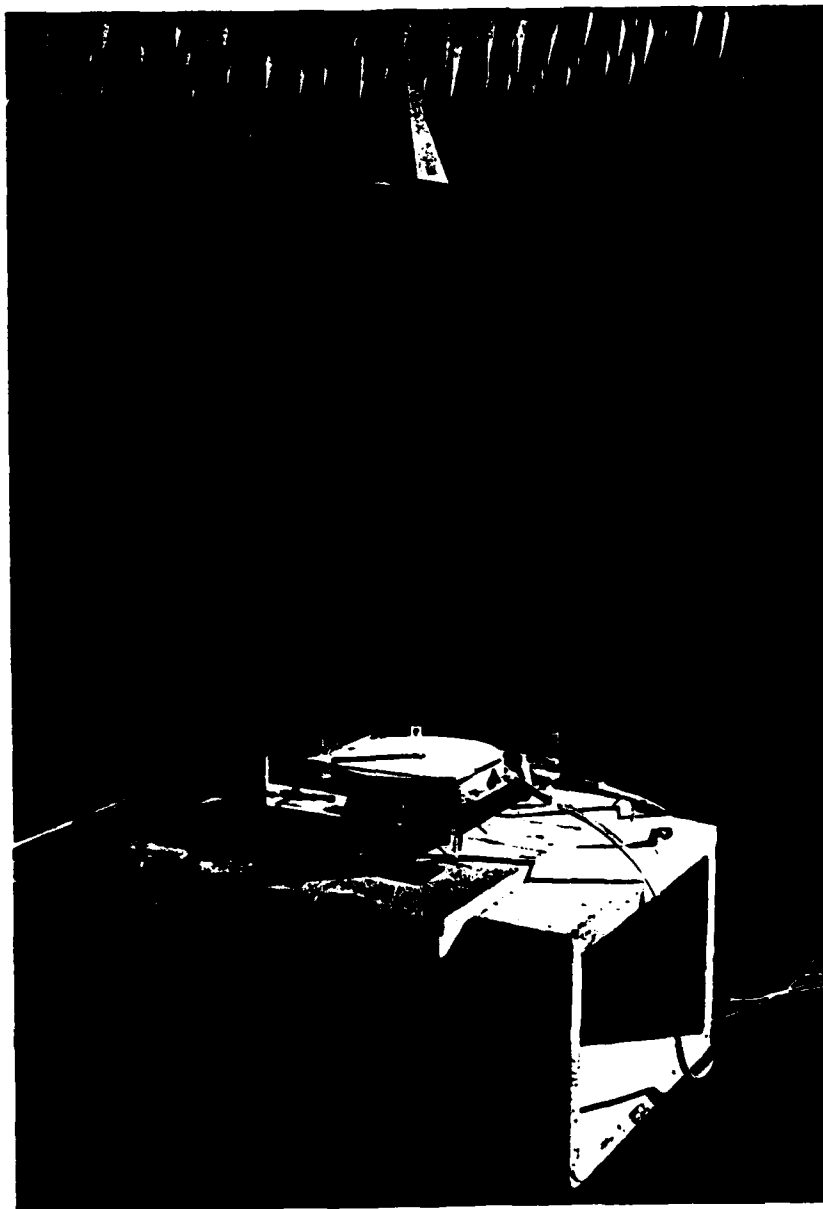


Figure 3. Horn-exposure water-bath geometry.

II. DETERMINATION OF POWER DENSITY DISTRIBUTIONS: 5.6 GHz(PW) AND 2.45 GHz(CW)

At the onset of this project, uncertainty existed as to which frequency and exposure mode would be studied. This uncertainty was due to a combination of: (a) transmitter availability (at desired frequencies); (b) depth of penetration of the microwaves into medium at those frequencies available; and (c) distance from horn to treatment flask at the available frequencies which would locate the treatment flask in the far field. Power density (PD) measurements were therefore first made at 5.6 GHz--a frequency which could meet our criteria--and, subsequently, at the more desirable 2.45 GHz.

A. 5.6 GHz, PULSE WAVE

A typical spectrum for the 5.6-GHz signal is shown in Fig. 4. In these exposures, the pulse width was 2 μ sec and the pulse repetition rate was 500 pps. Power densities were determined, at the surface of the Styrofoam^R exposure table, at nine points within the dimensions of the water bath. A Narda Probe (Model 8326B) was used; the forward power was 800 W. The probe was positioned parallel to the H field. The power density at each point was measured three times. A nine-orientation power density measurement was then made at the central point, and these data were averaged to yield an "absolute" power density. All data were corrected for probe variability and then normalized to the "absolute" power density.

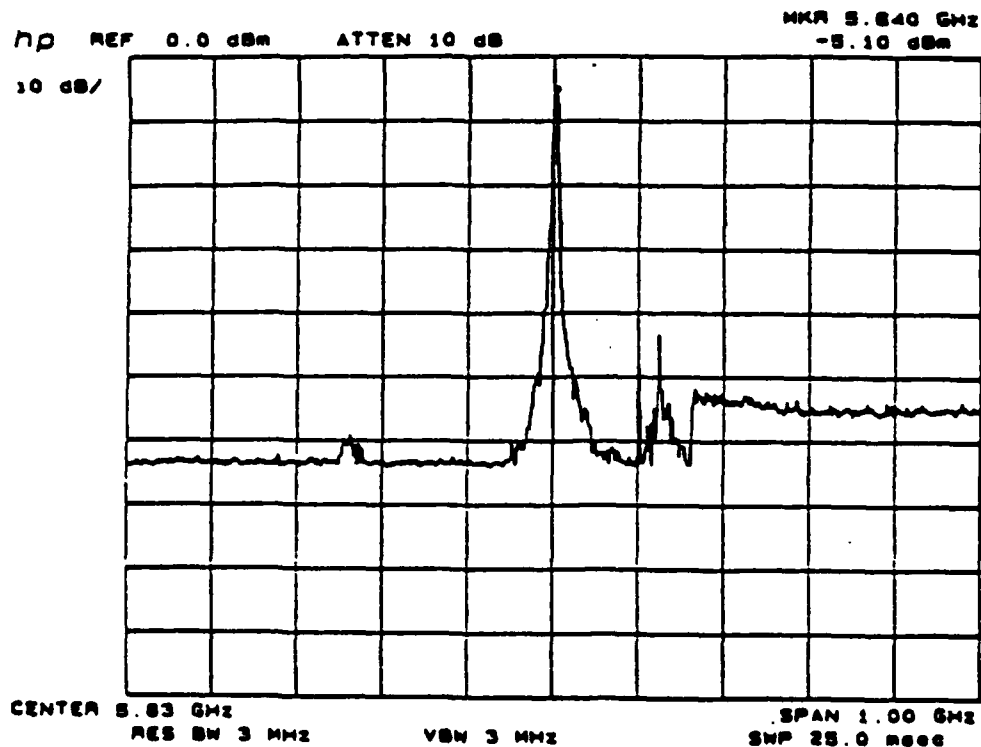


Figure 4. Signal quality plot of a typical spectrum at 5.6 GHz.

The means and standard deviation for the nine positions are presented in Fig. 5. The mean power density for the field was 324 ± 60 mW/cm². The up to 19% variation about the mean is consistent with our previous experience.

B. 2.45 GHz, CONTINUOUS WAVE

The power densities at 2.45 GHz were determined in a manner similar to that just described, except that the handle of the probe was positioned parallel to the K direction (the direction of wave propagation). A Narda Probe (Model 8623) was used; the forward power was 500 W. For the 2.45-GHz exposures, the 3-dB point bandwidth is 220 kHz and the second harmonic is 40 dB down from the primary frequency.

The power density at each point was measured once, with the probe oriented in one direction. In the center of the field, measurements were taken with the probe oriented in nine directions. These values were averaged and then corrected by the probe specific factor (0.934), yielding an average power density of 42.6 at the field center. The correction factor for the single measurements was therefore $42.6 \div 45.3 = 0.94$. (The corrected values are presented in Fig. 6.) The variation about the mean ranged up to 17%. The mean power density for the field was 36.3 ± 3.7 mW/cm².

III. SPECIFIC ABSORPTION RATE (SAR) DETERMINATIONS

The specific absorption rates (SAR) of culture medium in each of the ten T-25 culture flasks were determined as described previously (Meltz (1); Lozano (2)), with the assistance of the technical staff at the USAF School of Aerospace Medicine.

Briefly, 10 ml of the Eagles Basal medium (BME) to be used in the biological experiments was pipetted into each T-25 flask. (A hole had previously been drilled through the top plastic surface in the center of each flask.) The SAR determinations were performed in anechoic chamber No. 1 (Building 1187, Radiofrequency Radiation Research Laboratory, USAF School of Aerospace Medicine). The chamber temperature was maintained at 37°C. Vitek probes were positioned in four of the T-25 flasks, and an additional probe was placed in the exposure water bath.

The vitek meters were interfaced to a Hewlett Packard 9803A computer for printout of the temperature measurements. The circular float was kept stationary during the measurements.

These measurements were taken at 10-sec intervals during the stabilization period prior to RFR exposure, the RFR exposure interval, and the subsequent cool-down period after the RFR power was turned off. Heating and cooling data were collected 4 times in each position. The four probes were rotated to the different flask positions after each series of measurements. The measured temperatures during heating and cooling were then entered into an SAR computer program with a correction factor to determine the SARs at the exposure level of 10 mW/cm².

315	270	350
322	271	362
323	273	346
Avg = 320 ± 4.4	Avg = 271 ± 1.5	Avg = 353 ± 8
270	230	315
280	242	322
282	233	307
Avg = 277 ± 6	Avg = 235 ± 6	Avg = 315 ± 8
365	325	433
380	328	430
400	336	426
Avg = 382 ± 18	Avg = 330 ± 6	Avg = 430 ± 4

Figure 5. Power density map for 5.6 GHz, pulse wave, 500 pps, duty factor 0.001, 800-W forward power. All values are expressed in mW/cm^2 .

33.8	39.5	33.8
35.7	42.6	35.7
32.9	40.4	32.0

Figure 6. Corrected power density map for 2.45 GHz, continuous wave, 500-W forward power. All values are expressed in mW/cm^2 .

A. 5.6 GHz

The forward power level at 5.6 GHz was 800 W, thus resulting in a measured power density of 235 mW/cm² at the surface of the exposure table and at the center of the field. The SAR data for 5.6 GHz are summarized in Fig. 7. The mean SAR for all points is 110 ± 19 mW/g. A variation of up to 17% exists between the mean and individual positions. This variability will be averaged out by rotating all flasks about the center of the field during exposure.

B. 2.45 GHz

The specific absorption rates (SARs) at 2.45 GHz were determined as just described, with data collected twice at each position. The forward power level of 500 W resulted in a measured power density of 42.6 mW/cm² at the surface of the exposure table and at the center of the field.

The SAR data at 2.45 GHz are summarized in Fig. 8. The mean SAR for all points is 10.5 ± 1.0 mW/g. A variation of up to 16% exists between the mean and individual positions. This variation may be due to differences in the depth of the probe tip in the medium during different measurements.

	114 \pm 14	120 \pm 9
	119 \pm 7	92 \pm 39
Chamber Door	142 \pm 14	119 \pm 46
	123 \pm 30	80 \pm 22
	103 \pm 30	90 \pm 38

Figure 7. Specific absorption rate map - 5.6 GHz. All values expressed as mW/g. Each point represents one flask position in the circular holder.

	8.5 ± 0.2	11.6 ± 0.9
	10.0 ± 0.3	10.0 ± 0.2
	M = 9.25	M = 10.80
	8.8 ± 0.2	10.8 ± 0.9
	9.9 ± 0.5	10.3 ± 0.1
	M = 9.35	M = 10.55
Chamber	11.0 ± 0.3	11.1 ± 0.7
Door	9.5 ± 0.3	10.0 ± 0.2
	M = 10.25	M = 10.55
	11.7 ± 0.2	9.1 ± 0.5
	11.8 ± 0.3	12.7 ± 0.5
	M = 11.75	M = 10.9
	12.0 ± 0.5	10.0 ± 0.3
	12.3 ± 0.6	8.0 ± 0.5
	M = 12.15	M = 9.0

Figure 8. Specific absorption rate map - 2.45 GHz. All values are expressed as mW/g. Each point represents one flask position in the circular holder. Individual values and their respective means (M) are given.

IV. TEMPERATURE PATTERNS IN SIMULATED RFR EXPOSURE SITUATIONS AT 5.6 GHz (PW) WITH THE ANECHOIC CHAMBER AT AMBIENT TEMPERATURE AND HUMIDITY

A. OBJECTIVE:

The objective of this experiment was to evaluate the pattern of temperature increase in the liquid in an exposure (T-25) flask under experimental RFR exposure conditions. The irradiation exposure water bath geometry to be used in future RFR experiments was employed, and exposures were performed at increasingly higher power densities which resulted from higher transmitter forward power outputs.

B. METHODS:

1. Exposure Parameters

- a. Chamber description: Anechoic chamber in Bldg. #1185 at ambient temperature and humidity
- b. Transmitter-amplifier: Cober Electronics, Inc., Peak Power Emission Simulator, Model 2852
- c. Wave-guide connection to horn (description): Approximately 5 m of various C-band wave guide elements.
- d. Horn description and dimensions: Narda Microline, Model 848, Serial #7210
- e. Horn to flask distance: 1.4 m
- f. Frequency: 5.6 GHz
- g. Mode: Pulsed wave
- h. Repetition rate: 500 pps
- i. Pulse width: 2 μ sec
- j. Duty factor: .001
- k. Average forward power: 50-600 W
- l. Time between repeated temperature readings: 27.6 sec
- m. Flask position monitored: No.2
- n. Heater-circulator used:
Model: T.E.V. 45/100 Neslab Instrument Inc.

2. Monitoring Equipment

- a. Power Density Meter: Narda Probe Model 8326B
- b. Wave Form Quality: Photo Available (see Fig. 4).

3. Procedure

In the standard protocol, the heater-circulator pump was turned on, and the exposure system temperature was allowed to stabilize. Under the defined physical conditions employed (i.e., ambient anechoic chamber temperature and humidity, tubing length, pump used, etc.), a temperature of 37°C in the liquid in the treatment T-25 flask in the exposure water bath was established. A flask containing 12 ml of medium was placed in each position in the Styrofoam wheel holder. The flasks were rotated during the entire experiment.

After equilibration was achieved, and the baseline value of 37°C was evident on the plotter, the desired forward power was transmitted to the horn. Forward powers of 50 , 100 , 200 , 300 , 400 , 500 , and 600 W were measured by use of a bidirectional coupler and Hewlett Packard power meter at the output of the transmitter, prior to transmission through a waveguide connected to the horn antenna.

The flasks were exposed in the far field. The exposures at different power levels were performed on different days. A 500-W exposure was performed on the first day (Day A) and the 50-W exposure on a second day; the 100-, 200- and 300-W exposures were performed on a third day, and the 400- and 600-W exposures on a fourth day.

The RFR exposures were continued until the temperature in the treatment flask, exposure water bath, and circulator-heater had approached their peak values. This procedure typically required 30-50 min. At that time, the power was turned off, and the monitoring of temperature ceased.

C. RESULTS

The temperature patterns resulting from the heating (Figs. 9-16) are summarized in Fig. 17. In each figure, the uppermost solid line (if it has a 0 time intercept near 41°C) is the temperature measured in the heater-circulator. The dashed line, beginning with a 0 time temperature of 37°C, is the measured temperature in the treatment flask. The dash-dot line, likewise beginning with a 0 time temperature at 37°C, is the measured temperature in the exposure water bath.

Fig. 9 is the plot for temperature stabilization of the experimental system on one experimental day, with the anechoic chamber at ambient temperature and humidity. On each experimental day, the system was similarly allowed to come to equilibrium. On a second experimental day, with the same settings on the heater-circulator resulting in a similar temperature in the heater-circulator bath, a significantly higher temperature (0.5°C) was measured in both the exposure water bath and the flasks. This was compensated for by lowering the temperature of the heater-circulator as required to bring the flask temperatures to 37°C at equilibrium.

Shown in Figs. 10-16 are the temperature increase patterns for 5.6 GHz RFR pulsed wave, at the forward power levels (measured at the transmitter) of 50 , 100 , 200 , 300 , 400 , 500 , and 600 W. These data are summarized in Fig. 17, in which is shown the rapid rise of temperature within the first 20 min of RFR exposure, and the subsequent approach to equilibrium at 40 min.

The highest temperature achievable in the treatment flask at the highest power setting, 600 W, with a duty factor of .001, was 41.6°C. At the lowest power output tested, 50 W, an increase of temperature in the flask to 37.4°C was observed.

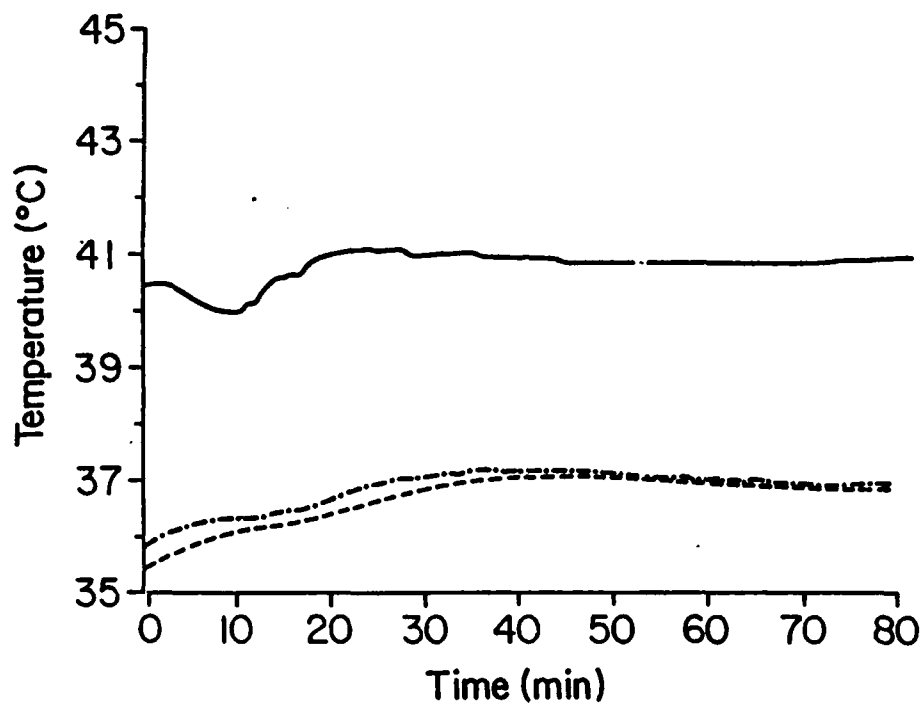


Figure 9. Temperature heating pattern in the exposure flask (----), exposure water bath (-·-·-·-·-), and heater-circulator (—), during the equilibration period before RFR exposure.

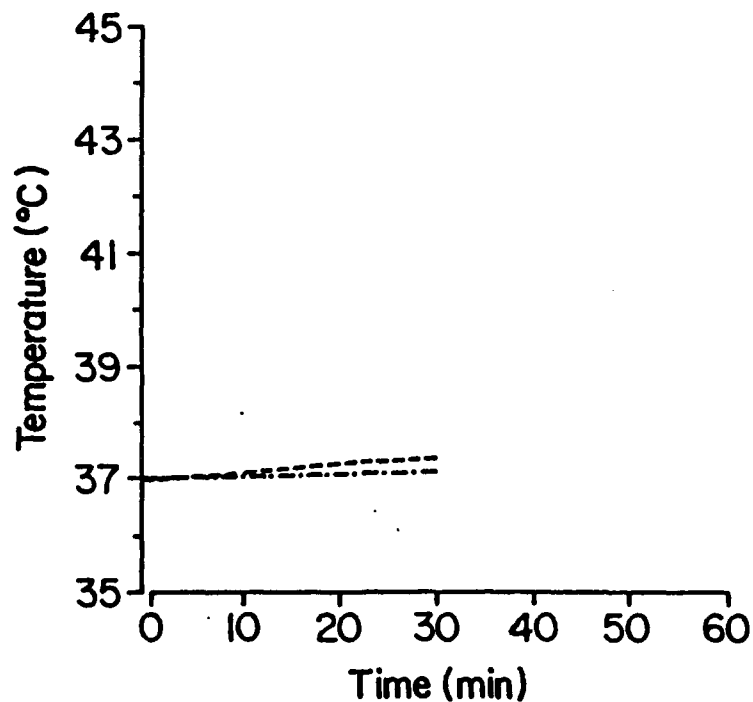


Figure 10. Temperature heating pattern in the exposure flask (----) and exposure water bath (- - - - -), during a 30-min exposure to 5.6-GHz pulse-wave radiation at 50-W forward power.

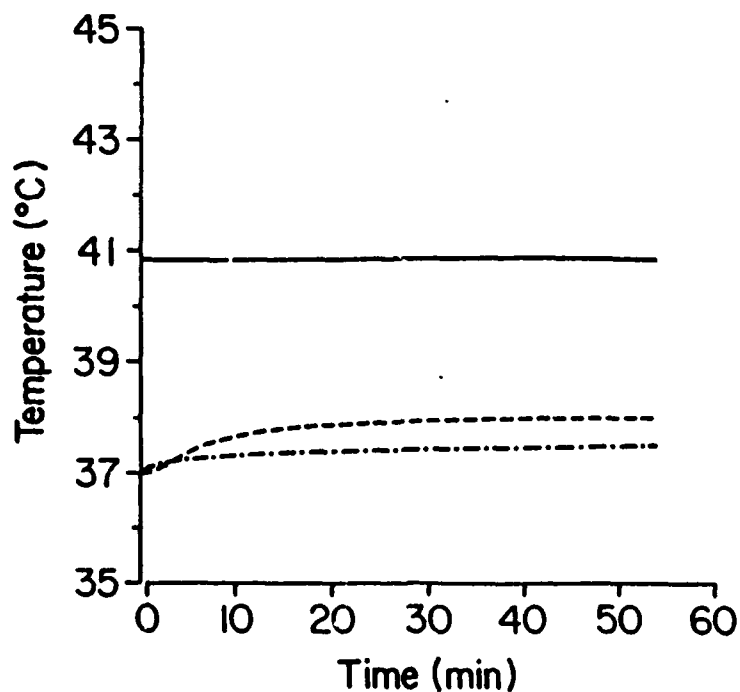


Figure 11. Temperature heating pattern in the exposure flask (----), exposure water bath (- - - - -), and heater-circulator (—), during a 50-min exposure to 5.6-GHz pulse-wave radiation at 100-W forward power.

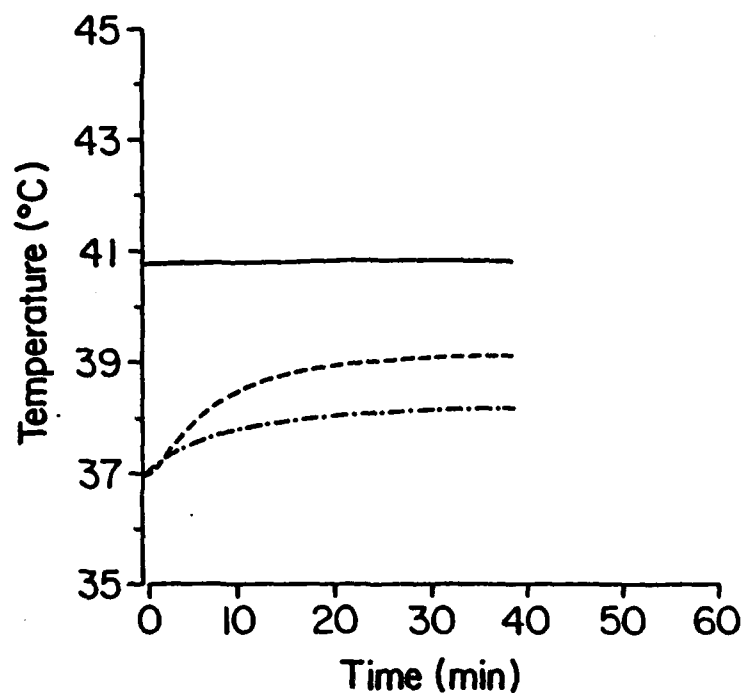


Figure 12. Temperature heating pattern in the exposure flask (----), exposure water bath (- · - · - · -), and heater-circulator (—), during a 40-min exposure to 5.6-GHz pulse-wave radiation at 200-W forward power.

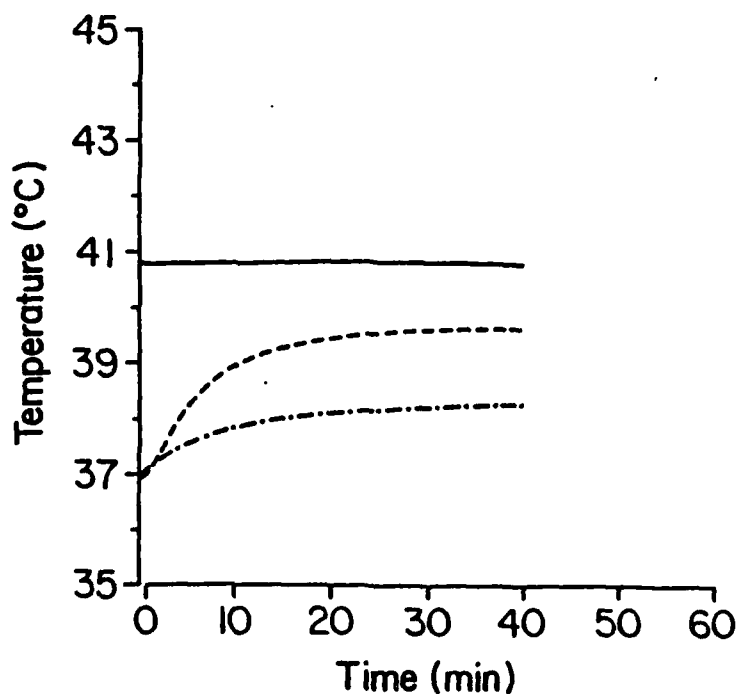


Figure 13. Temperature heating pattern in the exposure flask (----), exposure water bath (- · - · - · -), and heater-circulator (—), during a 40-min exposure to 5.6-GHz pulse-wave radiation at 300-W forward power.

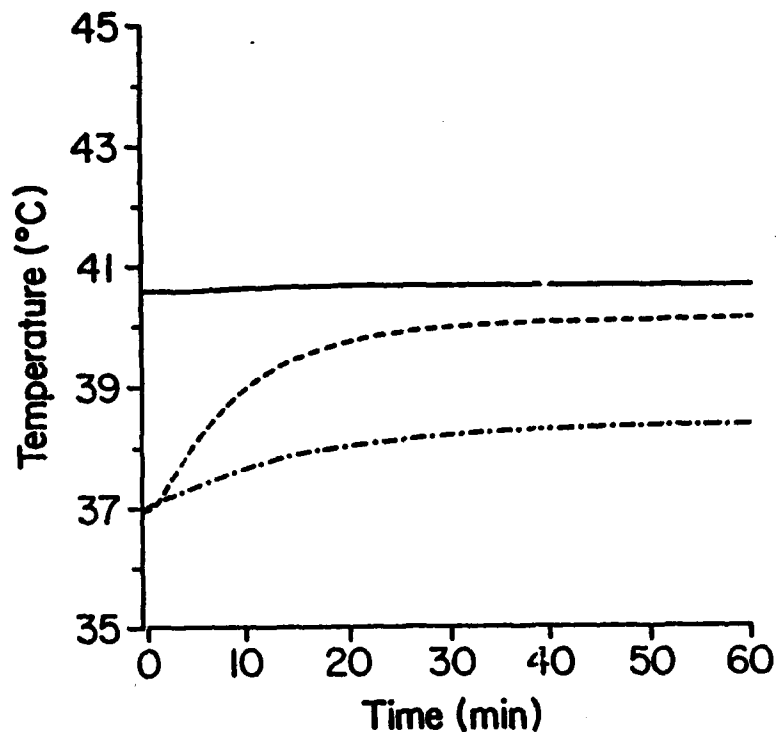


Figure 14. Temperature heating pattern in the exposure flask (----), exposure water bath (-.-.-.-), and heater-circulator (—), during a 60-min exposure to 5.6-GHz pulse-wave radiation at 400-W forward power.

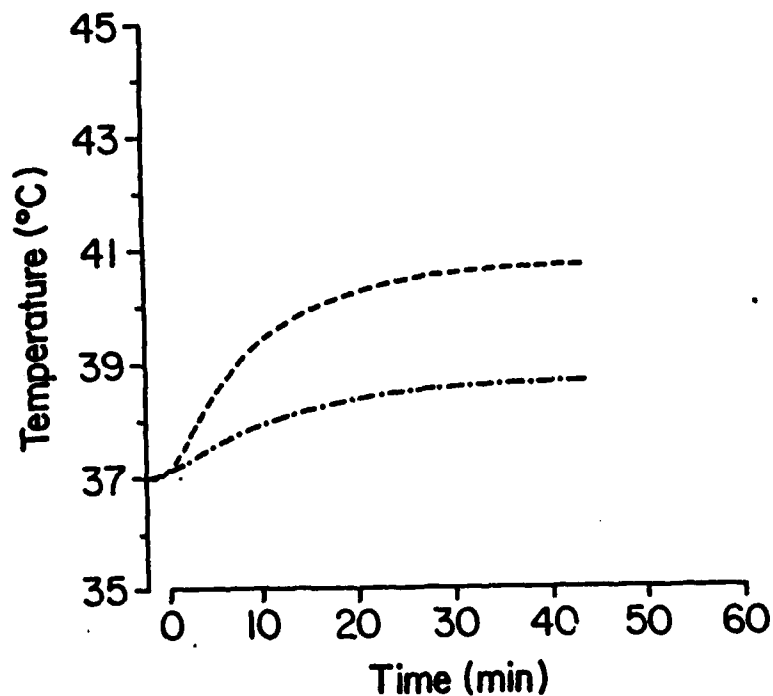


Figure 15. Temperature heating pattern in the exposure flask (----) and exposure water bath (-.-.-.-), during a 45-min exposure to 5.6-GHz pulse-wave radiation at 500-W forward power.

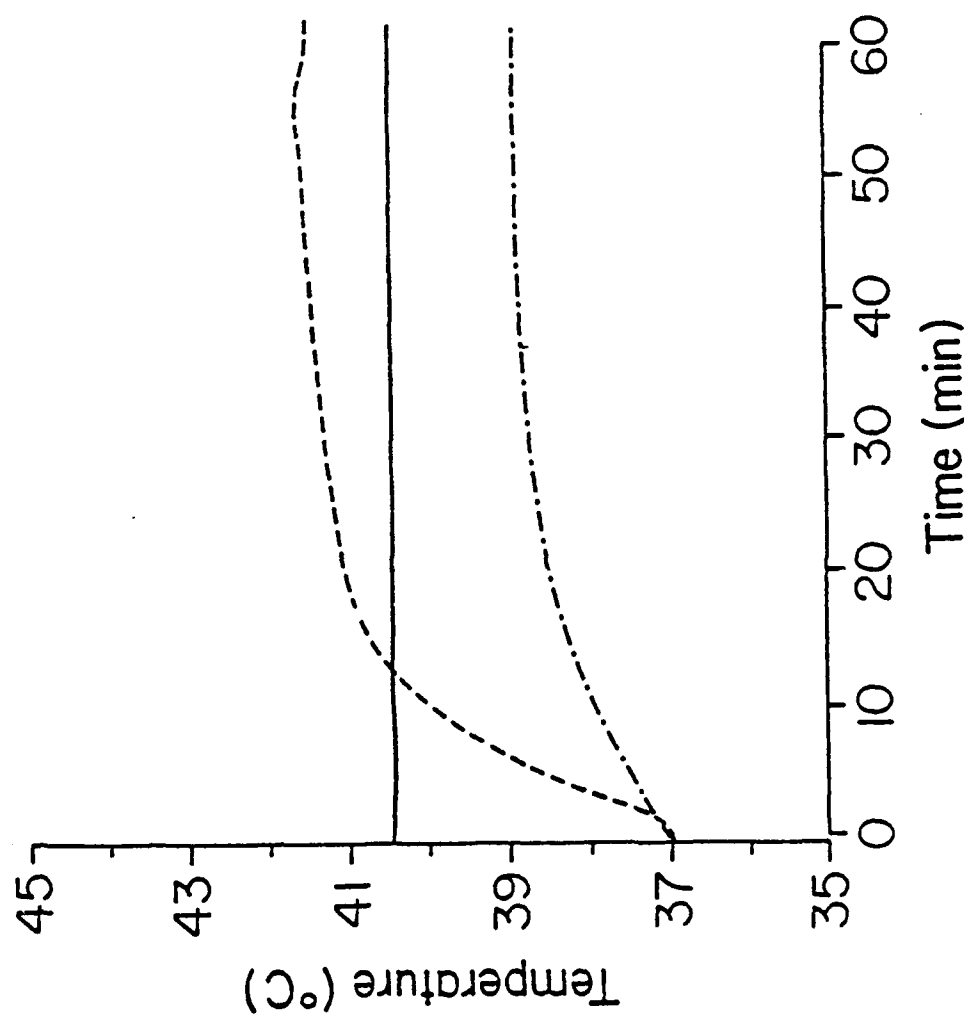


Figure 16. Temperature heating pattern in the exposure flask (-----), exposure water bath (-.-.-.-.), and heater-circulator (—), during a 45-min exposure to 5.6-GHz, P.W. radiation at 600-W forward power.

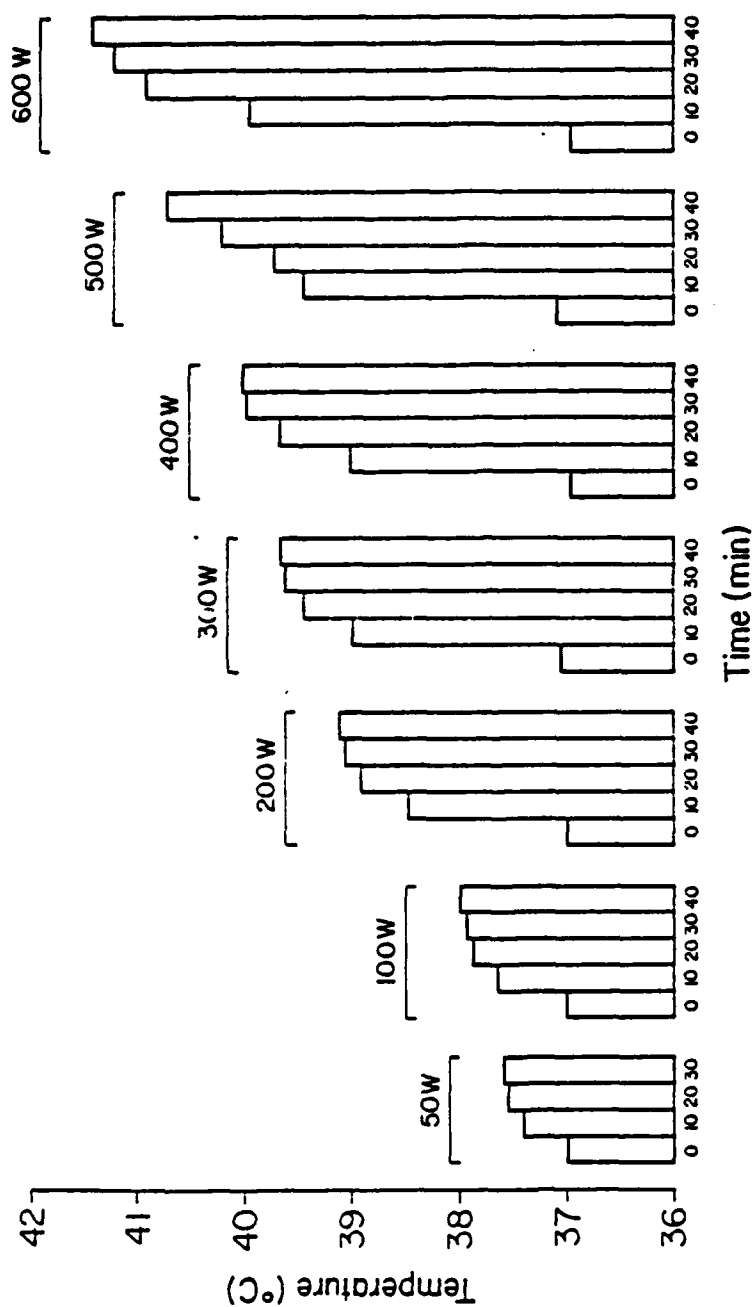


Figure 17. Compilation of temperature pattern data in the treatment flask at 10-min intervals for different power outputs at 5.6-GHz pulse-wave RFR exposure.

D. SPECIAL CONSIDERATIONS

As clearly shown in the preceding discussion (with respect to Fig. 9), allowing the anechoic chamber to remain at ambient temperature in these experiments is very undesirable; additional water-bath adjustments are required. We therefore decided that future experiments would be performed with the anechoic chamber equilibrated at $36.7^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$.

In addition, we observed that any disturbance in the rotation of the Styrofoam wheel supporting the flasks can lead to a temperature perturbation within the flasks. The rotation must be monitored during the RFR exposure.

V. TEMPERATURE PATTERNS IN SIMULATED RFR EXPOSURE SITUATIONS AT 2.45 GHz, CW, WITH THE ANECHOIC CHAMBER AT $36.8 \pm 0.2^{\circ}\text{C}$ AND AMBIENT HUMIDITY

The objective for this series of measurements is the same as just described for 5.6 GHz, PW Radiation.

A. METHODS

1. Exposure Parameters

- a. Chamber Description: Anechoic Chamber in Bldg. #1187 at $36.8 \pm 0.2^{\circ}\text{C}$ temperature and ambient humidity.
- b. Transmitter: Cober Electronics Inc., Stanford, Conn.
Model 1831 High Power Microwave Generator
- c. Cable connection to horn (Description):
Transmitter connected to horn via 7 m of 7/8 in. coaxial cable. Transmitter horn impedance was matched with a double-stub tuner.
- d. Horn description and dimensions: Struthers Electrical Corporation. Model 110N (Ser. No. 202); 28 x 37 cm.
- e. Horn to flask distances: 1.6 m
- f. Frequency: 2.45 GHz
- g. Mode: CW
- h. Repetition rate: Not applicable (N/A)
- i. Pulse width: (N/A)
- j. Duty factor: (N/A)
- k. Average forward power: 100-500 W
- l. Time between repeated temperature readings: 54 sec
- m. Flask position monitored: No.2
- n. Rotation speed of flasks in bath: (N/A)
- o. Heater-Circulator used:
 - (1) Company: Neslab Instrument Co.
 - (2) Model: T.E.V. 45/100

2. Monitoring Equipment

- a. Power density meter: Narda 8616
- b. Wave form quality: (N/A)
- c. Photo available: Yes No
- d. Temperature measurement: Vitek Model #101.

3. Procedure

The procedure employed is as just described for 5.6 GHz. The flasks were exposed in the far field. The exposure at different power levels were performed on different days. Exposures at 100 , 200 , and 300 W were performed on one day; 400- and 500-W exposures were performed three days later. The RFR exposures were continued until the temperature in the treatment flask, exposure water bath, and circulator-heater had approached near their peak values, typically 25-40 min. At that time, the power was turned off, and the monitoring of temperature ceased.

B. RESULTS

The temperature patterns resulting from the heating (Figs. 18-22), are summarized in Fig. 23. In each figure, the solid line (—) is the temperature measured in the heater-circulator. The dashed line (---), beginning with a 0 time temperature at 37°C, is the measured temperature in the treatment flask. The dash-dot line (-.-.-), likewise beginning with a 0 time temperature at 37°C, is the measured temperature in the exposure bath. The lower dashed line is the air temperature in the anechoic chamber. These data are summarized in Fig. 23, in which is shown a rapid rise of temperature within the first 10 minutes of RFR exposure, and the subsequent approach toward equilibrium at 40 min.

The highest temperature achievable in the treatment flask (containing 10 ml of medium), at the highest power setting, 500 W, was 38.3°C at 40 min. At the lowest power output tested, 100 W, an increase of temperature in the liquid in the flask to 37.6°C was observed at 40 min.

VI. DESCRIPTION OF THE L5178Y MOUSE LEUKEMIC CELL LINE

One of the cell lines employed in these studies is derived from the L5178Y mouse leukemic cell line, which was originally described by Fischer and Sartorelli (3). The specific cell line used in the assay, a heterozygote at the thymidine kinase (TK) locus, was isolated by Clive et al. (4) for use in the L5178Y thymidine kinase (TK +/-) locus mutation assay. Dr. Clive kindly provided us with the TK +/- cells, which have been grown and also frozen and stored under liquid nitrogen in our laboratory.

The cells are passaged twice weekly, by appropriate dilution, in fresh warm Fischers Medium for Leukemic Cells of Mice (Grand Island Biological Co.) with 10% heat-inactivated horse serum and the additives: sodium pyruvate (0.22 mg/ml); pluronic acid (1 mg/ml); and penicillin/streptomycin. An initial cell density after dilution of 5,000 or 10,000 cells/ml in a volume of 30 ml is typically used. The flask is gassed immediately after cell dilution with 5% carbon dioxide and 95% air to adjust the medium pH initially to 7.1 - 7.4. The cells are maintained in autoclaved, screwcapped, 125-ml glass flasks at 37°C on a rotating platform in a 37°C air incubator.

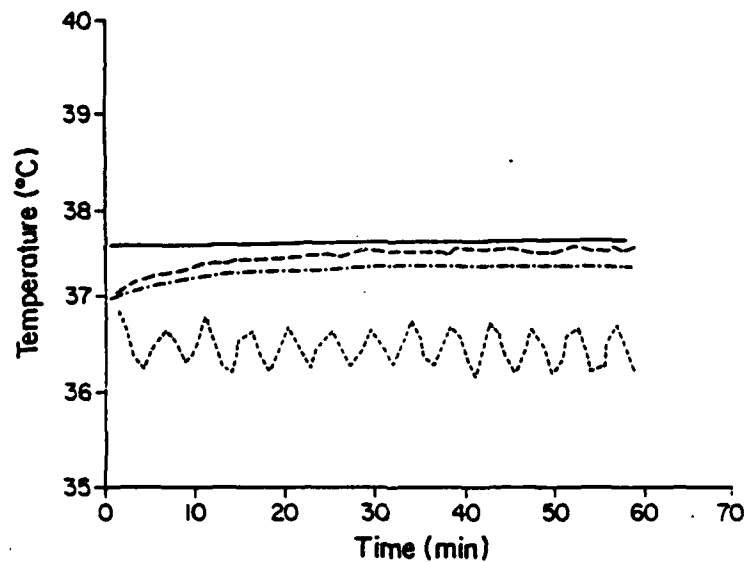


Figure 18. Temperature heating patterns in the heater-circulator (—), the treatment flask (---), the exposure bath (-.-.-.-), and the chamber air (----, lower line on chart), during a 60-min exposure to 2.45-GHz continuous-wave RFR at 100-W forward power.

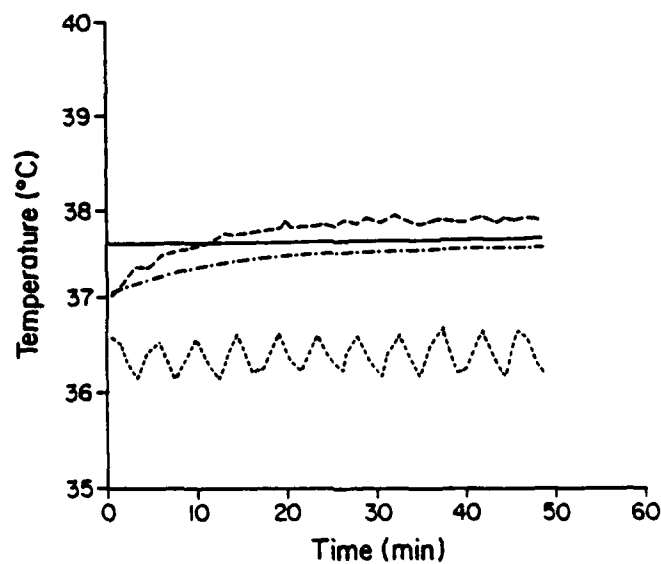


Figure 19. Temperature heating patterns in the heater-circulator (—), the treatment flask (---), the exposure bath (-.-.-.-), and the chamber air (----, lower line on chart), during a 50-min exposure to 2.45-GHz continuous-wave RFR at 200-W forward power.

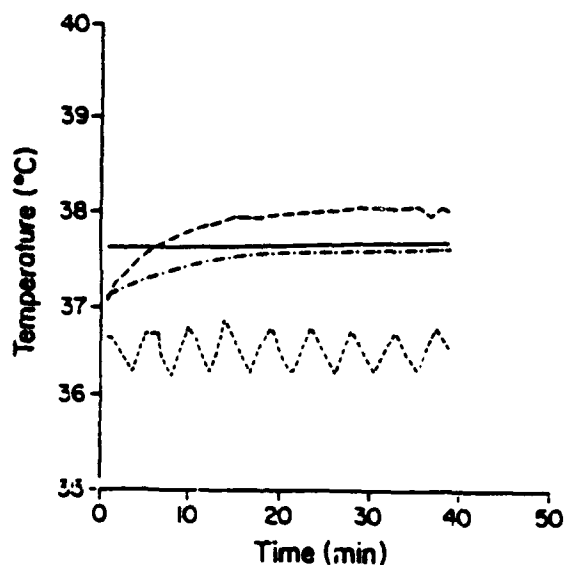


Figure 20. Temperature heating patterns in the heater-circulator (—), the treatment flask (---), the exposure bath (-·-·-·-), and the chamber air (----, lower line on chart), during a 40-min exposure to 2.45-GHz continuous-wave RFR at 300-W forward power.

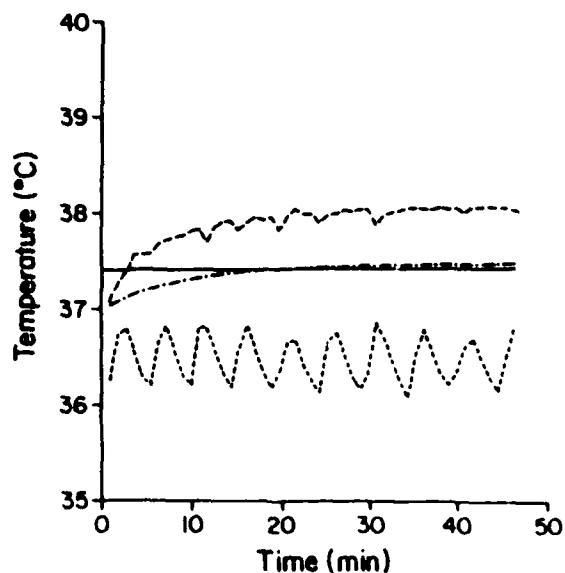


Figure 21. Temperature heating patterns in the heater-circulator (—), the treatment flask (---), the exposure bath (-·-·-·-), and the chamber air (----, lower line on chart) during a 50-min exposure to 2.45-GHz continuous-wave RFR at 400-W forward power.

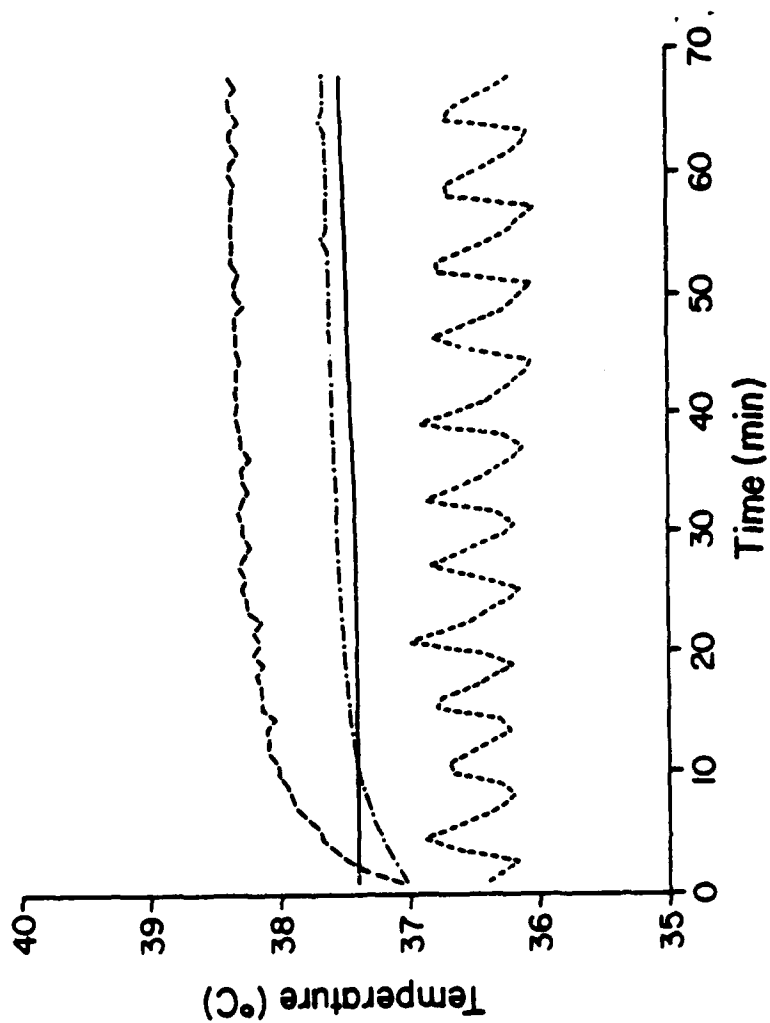


Figure 22. Temperature heating patterns in the heater-circulator (—), the treatment flask (---), the exposure bath (-.-.-), and the chamber air (.....), lower line on chart), during a 70-min exposure to 2.45-GHz continuous-wave RFR at 500-W forward power.

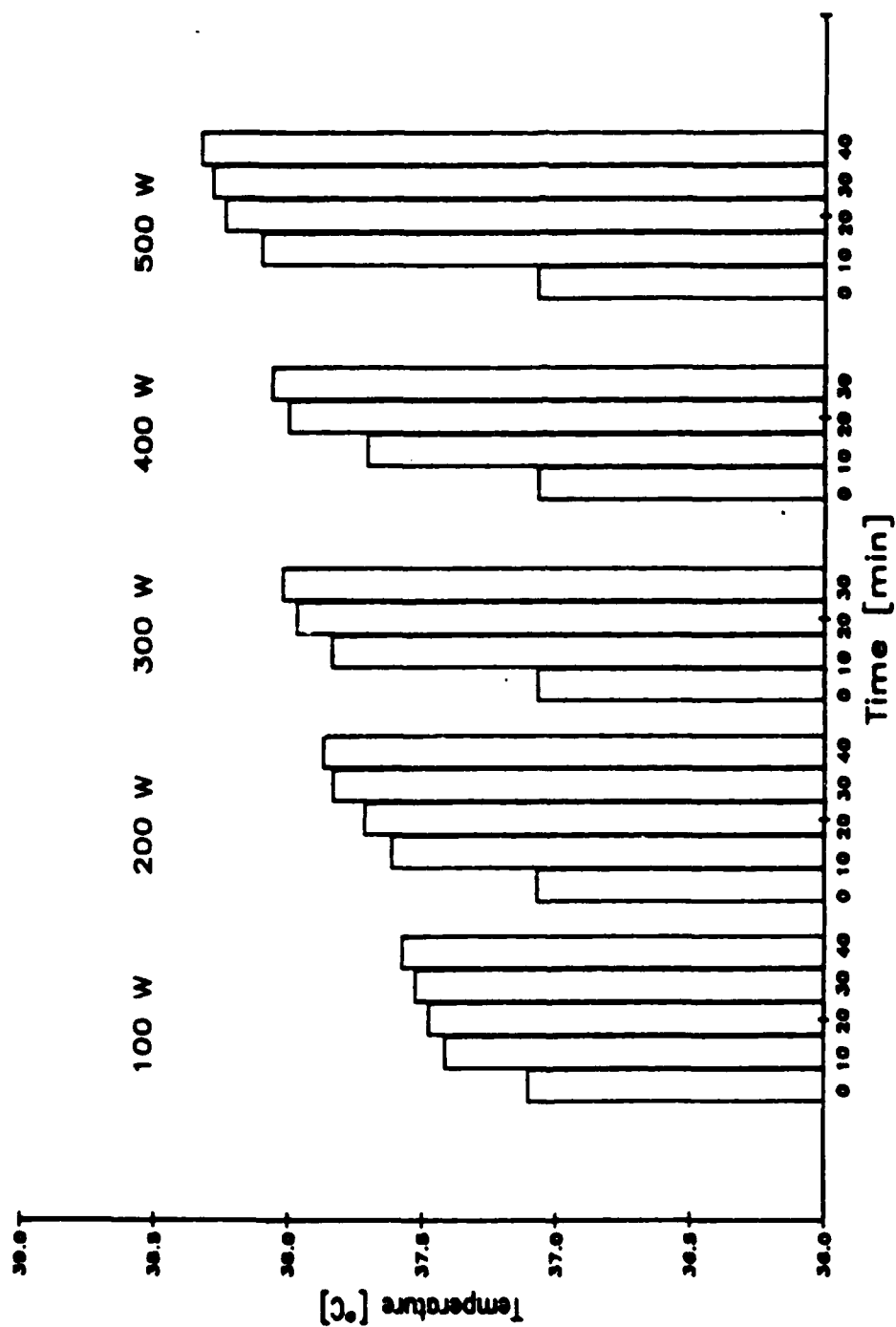


Figure 23. Compilation of temperature pattern data in the treatment flask at 10-min intervals for different power outputs at 2.45-GHz continuous-wave RFR exposure.

VII. CLONABILITY OF L5178Y CELLS IN SOFT AGAR

A. BACKGROUND

When soft agar cloning efficiency assays were performed in preliminary mutation experiments in our laboratory, the average cloning efficiency for non-treated control cells was lower than currently reported in other laboratories.

B. OBJECTIVE

Experiments were therefore designed to maximize the cloning efficiency of untreated "control" cells. Several variables were studied:

1. The Specific Autoclave Used for Sterilization

One autoclave used was a Magna-Clave, Model MC, located in laboratory 620F at the University of Texas Health Science Center in San Antonio (UTHSCSA). When used for sterilization, this autoclave was operated at 116°C for 20 min at 250 lb pressure. The second autoclave was a Sybron Barnstead Model C2250, located in the Radiofrequency Radiation Research Laboratory, U.S. Air Force School of Aerospace Medicine.

2. The Remelting Procedure:

Two procedures were used. Remelting of concentrated and previously autoclave-sterilized agar was performed on the day of cloning. In one procedure, the agar was remelted in an autoclave, either at UTHSCSA (116°C, 2 min, 240 lb pressure) or at USAFSAM (116°C, 2 min, variable pressure <150 lb). In the second, the agar was melted at the two different locations by placing a bottle with the previously autoclaved and gelled agar in a boiling water bath.

3. The Incubator Selection:

Two different incubators were used. One, located at UTHSCSA (Lab 621F), is a two-chamber Forma Scientific Vertical humidified CO₂ incubator (Model 3325). The second incubator, at USAFSAM, is a two-chamber Forma Scientific Model 3326 vertical CO₂ humidified incubator. Both incubators use 100% CO₂ gas cylinders supplied by a commercial vendor.

4. The Types of Agar :

Two types of commercial agar were studied. One was DIFCO Noble Agar (Lot #701844), regularly used in the L5178Y soft agar assay. The second was Key Noble Agar (Lot #60977).

C. METHODS

Four percent (4%) agar (DIFCO Noble or Key) in water, which had previously been sterilized in an autoclave at 116°C for 20 min and allowed to gel, was remelted both at UTHSCSA and USAFSAM by heating either (a) in an autoclave at 116°C for 2 min, or (b) in a boiling water bath until liquified. The agar was then distributed into flasks containing cloning medium, so that the final agar concentration was 0.4%.

A stock L5178Y cell suspension was next counted, and the cells distributed into the diluted agar preparations to yield a density of 6 cells/ml. At this time, the cell suspensions were distributed in 33-ml aliquots into 100-mm petri dishes, which were placed in a refrigerator freezer for 12 min to allow the agar to gel. The dishes were then placed in the laminar flow hood for 5 min to warm toward room temperature, before being placed in one of the 37°C humidified incubators. The procedures used at UTHSCSA and USAFSAM were identical, except as noted; and the cells came from the same cell stock on the same day.

D. RESULTS

The results are summarized in Table 1:

TABLE 1. AGAR CLONING STUDIES

Experiment Performed At:	Agar Brand	Agar Remelting Method	Individual Dish Percentage Cloning Efficiency	Average Percentage Cloning Efficiency
UTHSCSA	DIFCO	Water bath	47.4, 39.9, 40.4	42.6
UTHSCSA	DIFCO	Autoclave	38.4, 42.4, 32.3	37.7
UTHSCSA	KEY	Water bath	44.9, 41.9, 43.4	43.4
UTHSCSA	KEY	Autoclave	5.5, 13.6, 11.6	10.2
USAFSAM	DIFCO	Water bath	39.4, 33.3, 34.3	35.7
USAFSAM	DIFCO	Autoclave	33.8, 23.7, 27.3	28.3
USAFSAM	KEY	Water bath	1.5, 3.0, 2.5	2.3
USAFSAM	KEY	Autoclave	1.5, 1.5, 3.0	2.0

UTHSCSA - The University of Texas Health Science Center at San Antonio.

USAFSAM - USAF School of Aerospace Medicine, Brooks Air Force Base, Texas.

In each comparison, the cells grown in agar that had been remelted in an autoclave had a lower cloning efficiency than those cells grown in agar remelted in a water bath. This finding was particularly true when using Key Noble Agar, for which the cloning efficiency of cells in autoclave-melted agar (at UTHSCSA) was less than half that of cells in agar melted in a boiling water bath.

In every case, the DIFCO Noble Agar had equal or higher cloning efficiencies than the Key Noble Agar.

The plates placed in the incubator at UTHSCSA had higher cloning efficiencies than those incubated at USAFSAM.

E. CONCLUSIONS

The cloning efficiency of cells in agar is dependent on the method used for remelting. The use of a second autoclaving, even though for a shorter time (2 min) than used for sterilization (16 min), results in a decreased cloning efficiency. In all future experiments, the agar to be used on the cloning day will either (a) be remelted in a boiling water bath, or (b) be placed in a 60°C water bath immediately after autoclave sterilization (the first time).

The use of DIFCO Noble Agar clearly resulted in higher cloning efficiencies than when Key Noble Agar was used. The former, recommended in the standard protocol (4), is acceptable for this assay.

The cloning efficiency of the L5178Y cells in the agar plates incubated in the Forma Scientific incubator at UTHSCSA was consistently higher than that in the plates maintained in the Forma Scientific incubator at USAFSAM. The 100% CO₂ gas used in the incubators was from the same commercial supplier; no reason for the difference in measured cloning efficiency is available. The difference in cloning efficiencies is not of such a magnitude as to be of major concern; however, these values will not be pooled.

VIII. ADRIAMYCIN - PRELIMINARY STUDIES

A. OBJECTIVES

A series of preliminary investigations were undertaken with Adriamycin, a chemical known to damage DNA (5). These experiments were initiated to find:

1. The concentration range in which a 4-hr treatment of L5178Y cells with Adriamycin at 37°C would decrease cell proliferation over the subsequent 60-70 hr of growth, but still result in enough cells accumulating to perform a mutagenesis selection experiment after the 60- to 70-hr expression time;

2. At least two concentrations of Adriamycin where the inhibitory effect would not decrease cell survival below 10% (when the cells were cloned in soft agar after 60-70 hr of expression time),

3. Two concentrations where a clearly measurable increase in the induced mutant frequency would occur after a 4-hr treatment at 37°C and subsequent expression period of 60-70 hr.

B. METHODS

In the course of these experiments, the cells appeared to become "resistant" to the growth inhibitory activity of Adriamycin at previously active concentrations. Only one of these experiments is reported herein. Our action when this decreased activity was observed was to: rethaw previously frozen cell stock; and, in addition, obtain the line of LS178Y TK +/- cells currently being used by Dr. Don Clive at Burroughs-Wellcome Corp (who kindly forwarded the cells to us).

Also, in the course of these experiments, the handling of the cells immediately prior to addition of the chemicals was altered. In the earliest experiments, the cells were pelleted out of their growth medium and resuspended in completely fresh medium with 3% horse serum. In the more recent experiments, the cells were pelleted; but a volume of conditioned medium was retained, and this was diluted with fresh medium without serum to give a final concentration of 3% horse serum. These two methods and the post-treatment procedure are described next.

1. Complete Medium Replacement Method

The cells were first purified of spontaneous mutants by the standard sequential THMG/THG* treatment sequence (4). This procedure included a 64-hr incubation in medium-containing THG and, as necessary, dilution 24 hr prior to an experiment. On the experiment day, the density of the cells in the combined mass culture flasks was determined by using a Coulter Counter (Coulter Electronics, Inc.); and 6×10^6 cells were distributed in up to twelve 15-ml sterile conical centrifuge tubes. The cells were pelleted by centrifugation at 800 rpm (129 x g) for 8 min. The supernatant was aspirated; and the cells in each tube were resuspended in 10 ml of fresh warm Fischers medium with 3% horse serum (F_{3p}) [prepared by diluting F_{10p} with F₀]. The cell suspensions were transferred into T-25 flasks, and gassed with 5% carbon dioxide and 95% air. Immediately after this distribution, an appropriate volume of sterile water (0.1 ml), containing the concentrated Adriamycin (100 X the desired final concentration), was added to the flasks with a micropipetter. The experiment was carried out under yellow light. The closed flasks were placed in a light-limiting box in the rotating incubator for a 4-hr incubation at 37°C.

* T = thymidine M = methotrexate
H = hypoxanthine G = glycine

2. Partial Medium Replacement Method

In this modification, the cells in the previously grown (purified) mass cultures were combined and the cell density determined by using a Coulter Counter. That volume containing 6×10^6 cells \times the number of treatment flasks plus an additional 6×10^6 cells was calculated. This volume was then distributed equally into four 50-ml Corning centrifuge tubes and centrifuged (as stated in the preceding paragraph). The total volume of medium necessary to resuspend all of the cells at a final cell density of 600,000 cells/ml was calculated. All except 30% of this final desired volume was discarded; one-fourth of this total retained 30% volume was left in each of the four tubes. The cells were then resuspended in this conditioned medium, combined in a 250-ml Erlenmeyer flask, and brought up to the final total volume with F_0 , thus resulting in a cell density of 6×10^5 cells/ml in F_{3p} . The cell suspension was then allowed to equilibrate on a rotating shaker at 37°C for 10-15 min. After that time, 10-ml volumes of cells were distributed into treatment flasks, which were gassed for 1 sec with 5% carbon dioxide and 95% air and returned to rotation in the incubator for 5 min. These flasks were then removed to the hood, where 0.1 ml of the proper concentration of chemical (or control medium) was added to each. The flasks were immediately returned to the rotating incubator; treatment consisted of a 4-hr incubation at 37°C .

3. Post-Treatment Procedure For Growth Curve Determination

At the end of the 4-hr incubation, the cells were pipetted from the treatment flasks into sterile 15-ml conical tubes, and pelleted by centrifugation (750 rpm \times 8 min at room temperature). The supernatant containing the hazardous chemical was pipetted off and discarded; the cells were resuspended in fresh warm Fischers medium (F_{10p}) as a wash. They were then centrifuged and resuspended a second and third time in warm F_{10p} . A Coulter count was then performed on the cell suspension in the tube to determine the cell density.

On the basis of that information, the volume of cell suspension containing 1.2×10^6 cells was calculated. This volume of cell suspension was then added (for each of the 10 treatment flasks) into one or two 250-ml Corning Erlenmeyer flasks [gassed with 5% carbon dioxide and 95% air]; fresh F_{10p} was added to each flask so that the final volume of F_{10p} medium was 60 ml. The cell density in each case was approximately 20,000 cells/ml. For accuracy to be insured, the cell density of the 60-ml volume was redetermined by Coulter count after 30 min of equilibration in the rotating incubator at 37°C . The growth curve was established by performing replicate Coulter counts daily for each flask.

C. RESULTS

1. Low concentration range experiment (6 hr): 2.5×10^{-5} to 5×10^{-4} $\mu\text{g/ml}$.

Method used: Complete medium replacement.

The results from this experiment, performed with a 6-hr treatment with Adriamycin, showed no effect (Fig. 24).

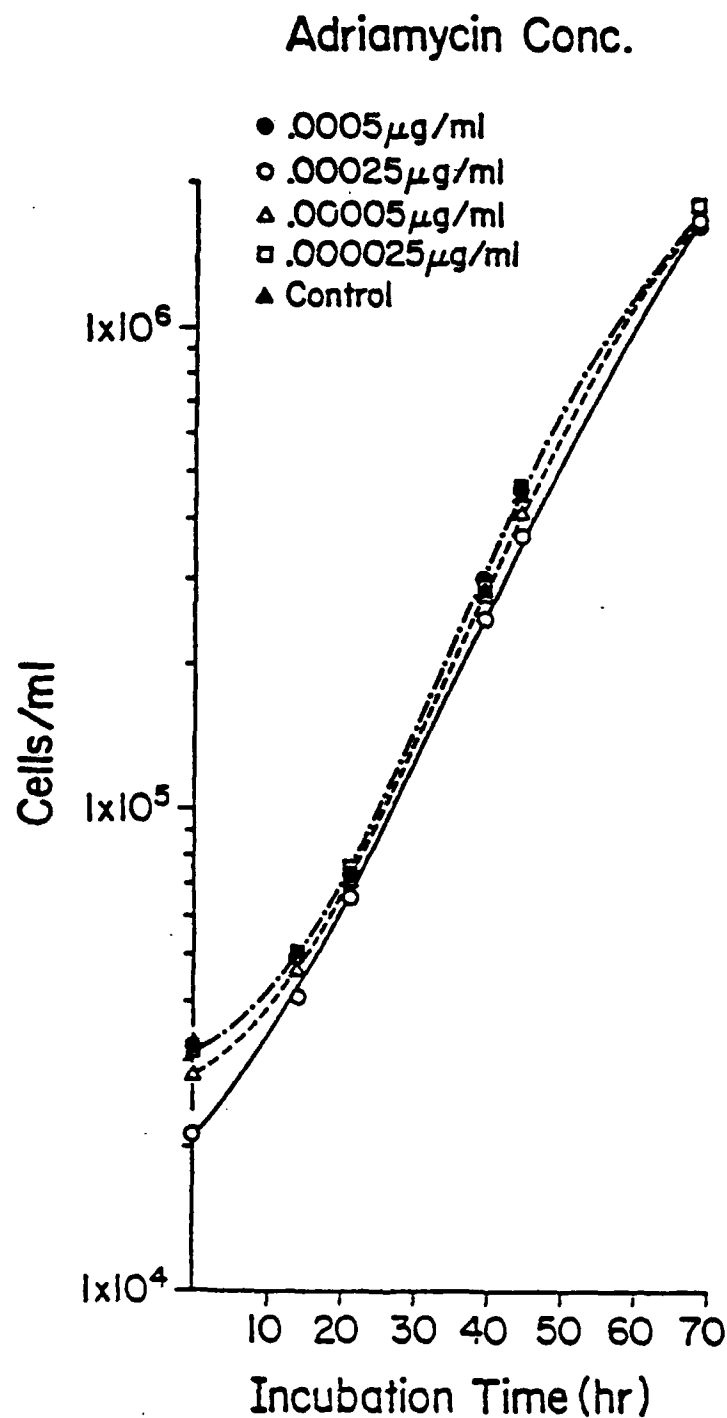


Figure 24. Growth of L5178Y cells after a 6-hr treatment with Adriamycin. The data are for individual growth curves initiated from one treatment flask at each chemical concentration, at a cell density of 20,000 to 30,000 cells/ml.

2. Adriamycin treatment (4 hr) at 0.005 to 0.1 $\mu\text{g}/\text{ml}$

Method used: Complete medium replacement.

The results of this experiment, performed with a 4-hr treatment, are shown in Fig. 25. To meet our objectives, repeating this experiment in the range between 0.01 and 0.05 $\mu\text{g}/\text{ml}$ was clearly necessary.

3. Adriamycin treatment (4 hr) at 0.01 to 0.05 $\mu\text{g}/\text{ml}$

Method used: Complete medium replacement.

The results of this experiment, performed with a 4-hr treatment, are shown in Fig. 26. They are consistent with our previous observations. This is the range from which one or two Adriamycin concentrations will be selected for mutagenesis experiments.

4. Adriamycin treatment (4 hr) at 0.005 to 0.04 $\mu\text{g}/\text{ml}$

Method used: Complete medium replacement.

In this experiment (shown in Fig. 27), the effect of Adriamycin was less than previously observed. As already mentioned, this observation led us to thaw new cells, and also to obtain a completely new cell stock from Dr. Clive.

IX. MITOMYCIN C - PRELIMINARY STUDIES

A. OBJECTIVE

This experiment was performed in the concentration range of 0.1 $\mu\text{g}/\text{ml}$ - 1.0 $\mu\text{g}/\text{ml}$. It was designed to allow selection of a moderately toxic concentration of mitomycin C for subsequent mutagenicity tests.

B. METHOD

The method employed in this experiment was the partial medium replacement method just described.

C. RESULTS

The results of this experiment, performed with a 4-hr treatment, are presented in Fig. 28. They clearly show the growth inhibitory effects of a 4-hr treatment with mitomycin C at increasing concentrations. Based on these studies, we will select chemical concentrations no greater than 0.5 $\mu\text{g}/\text{ml}$ and no less than 0.05 $\mu\text{g}/\text{ml}$ of mitomycin C for preliminary mutagenicity testing.

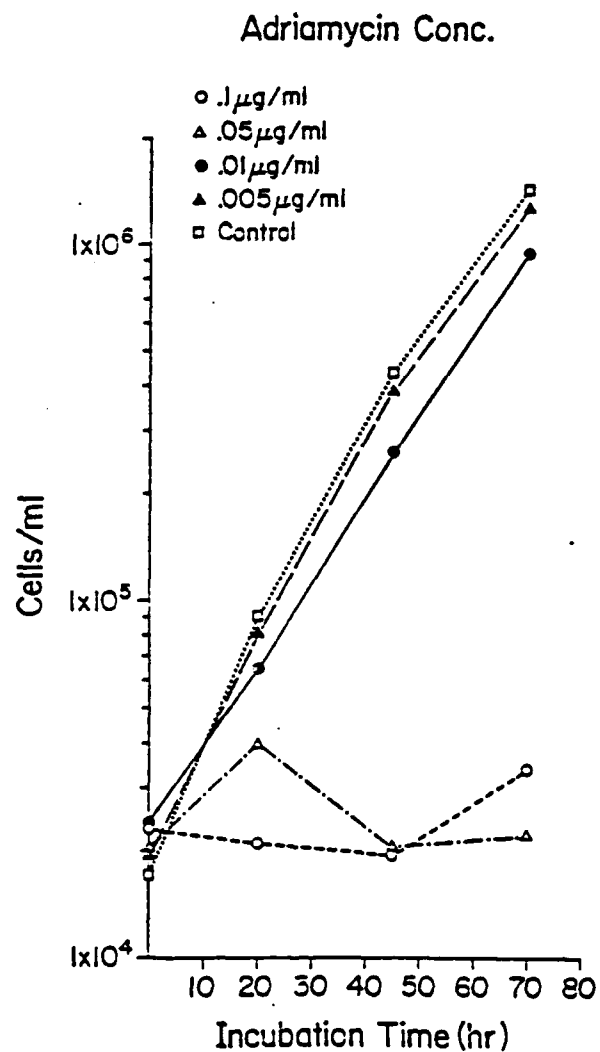


Figure 25. Growth of L5178Y cells after a 4-hr treatment with Adriamycin: .005-0.1 µg/ml. The data are for individual growth curves initiated from one of the treatment flasks at each chemical concentration.

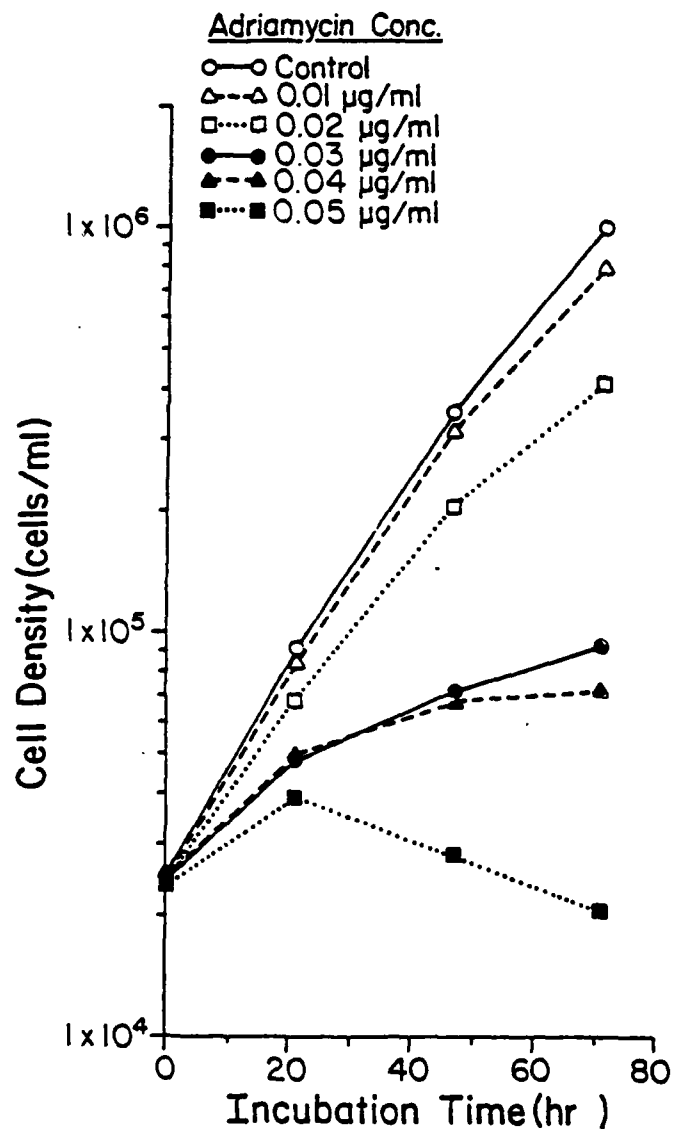


Figure 26. Growth of L5178Y cells after another 4-hr treatment with Adriamycin: 0.01-0.05 $\mu\text{g/ml}$. The data are for individual growth curves initiated from one of the treatment flasks at each chemical concentration.

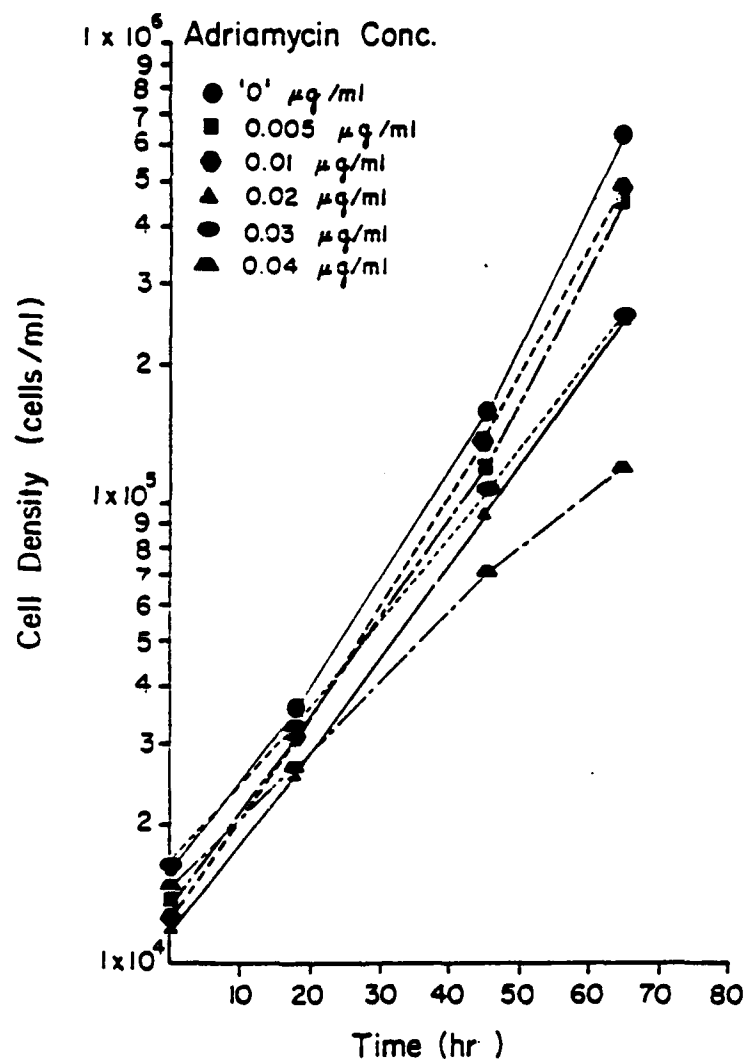


Figure 27. Growth of L5178Y cells after the same 4-hr treatment with Adriamycin: 0.005-0.04 $\mu\text{g/ml}$. The data are for individual growth curves initiated from one of the treatment flasks at each chemical concentration, at a cell density less than the standard 20,000 cells/ml.

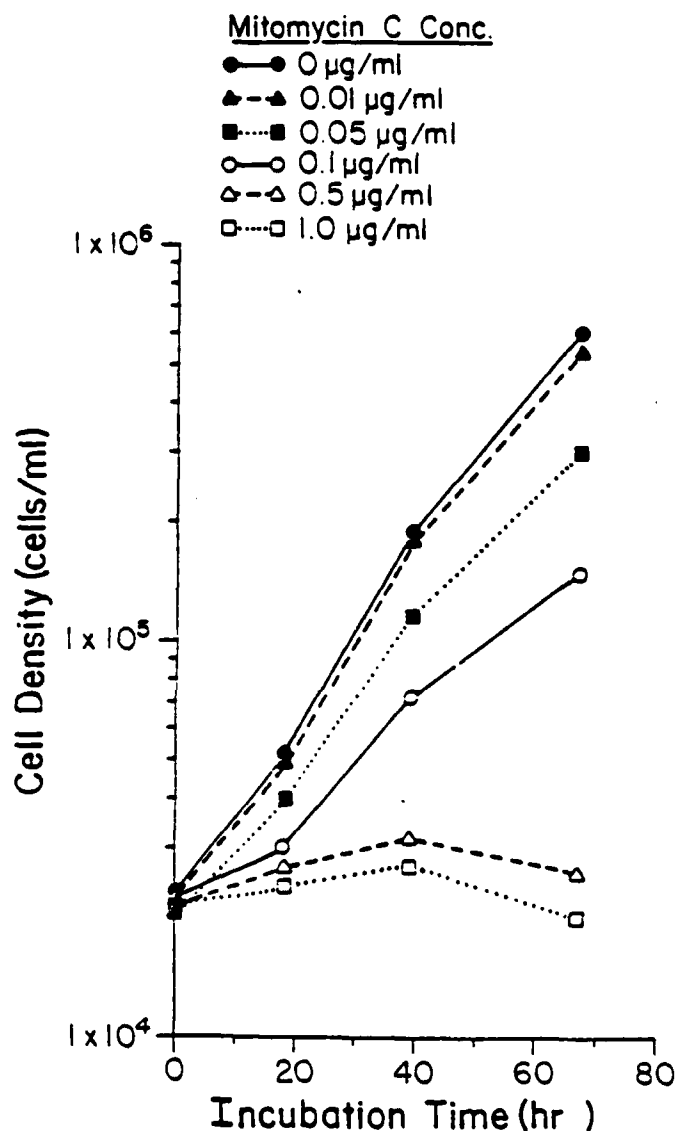


Figure 28. Growth curve of L5178Y cells after a 4-hr treatment with mitomycin C at different concentrations. The data are averaged for four different growth curves initiated (two each) from two different (independent) treatment flasks at each chemical concentration tested.

X. EFFECT OF CONVECTION-INDUCED HEAT TREATMENT, OVER THE TEMPERATURE RANGE OF 37°C - 45°C, ON L5178Y CELL GROWTH.

A. OBJECTIVE

RFR studies at higher power levels would be expected to result in a temperature increase in the medium over the 4-hr exposure interval to be used. Before initiating these RFR studies, the effect of incubating the L5178Y cells at different temperatures on their subsequent growth was studied. Heating was achieved by convection (water-bath) heating. The results of this experiment would enable us to set an upper limit on the RFR power levels subsequently employed, since preliminary studies of changes in medium temperature at increasing power levels can readily be performed.

B. METHODS

The L5178Y cells were first purified of spontaneous mutants by the standard THMG-TEG treatment sequence. The density of the cells in the mass culture flask was then determined, and cells were distributed into twelve T-25 flasks at a final cell density of approximately 600,000 cells/ml. Replicates were performed at each temperature; two additional flasks were incubated at 37°C. The complete medium replacement method, described previously, was used.

The flasks were immersed in water baths which had already been equilibrated at 37°C, 39°C, 41°C, 43°C, and 45°C. The flasks remained stationary, with occasional swirling by hand during the 4-hr incubation.

Subsequent to the 4-hr incubation at the various temperatures, the cells were transferred into tubes, washed, resuspended, and distributed into final 60-ml volumes of F10p medium with 10 mM [N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid] (HEPES) in 250-ml flasks. The initial cell density was approximately 20,000 cells/ml.

C. RESULTS

The results are presented in Fig. 29. While temperature control was not as accurate (+ 0.5 degrees) as will be employed in RFR studies, the results of this experiment are consistent with those obtained for a wide variety of mammalian cell lines.

After incubation for 4 hr at temperatures up to 41°C, subsequent cell growth at 37°C appears relatively unaffected. Any temporary lag in cell growth which may have occurred in the initial hours after the 4-hr incubation at 39°C and 41°C cannot be verified, because an initial post-dilution cell count was not performed. Any such lag in this temperature range will, however, have to be taken into account, because it can affect the results of the sister-chromatid exchange measurements in future experiments to be performed with CHO cells.

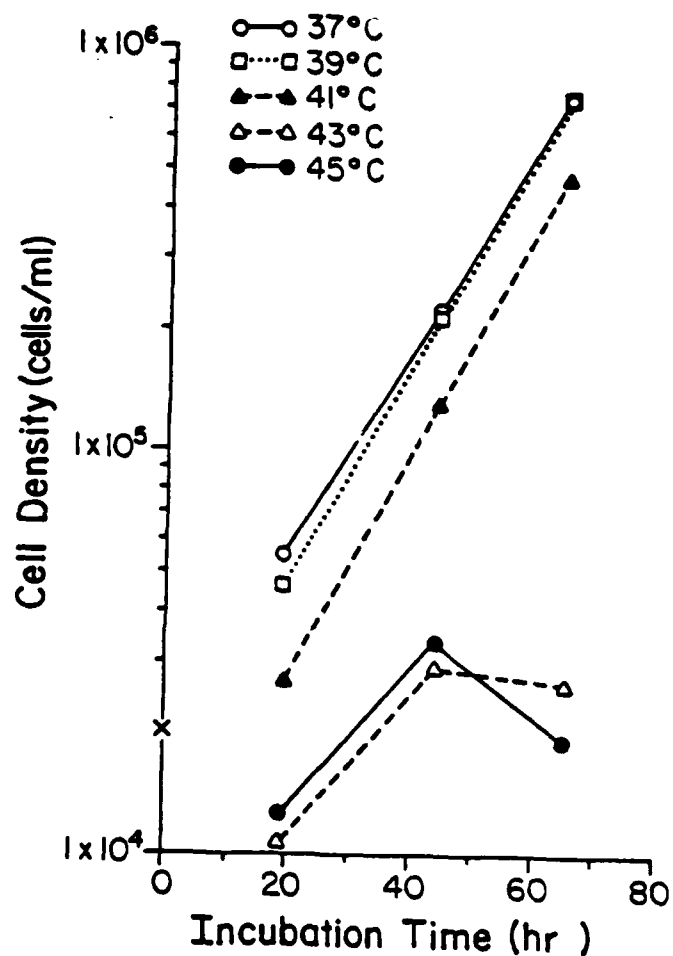


Figure 29. Growth curve of L5178Y cells after a 4-hr incubation at different temperatures. The data are averaged for two different growth curves, initiated from two independent treatment flasks at each temperature studied.

At 43°C and 45°C, obvious inhibition of cell growth occurred. Cell loss also appears to have occurred immediately after the 4-hr incubation. This possibility will have to be confirmed by hemocytometer count, since dead cells still present in the suspension will not be counted by the Coulter counter.

XI. ETHYLMETHANE SULFONATE (EMS) MUTATION EXPERIMENT

A. OBJECTIVE

To verify the mutagenic response of the L5178Y mouse Leukemic cell thymidine kinase locus mutation assay system, EMS (a known active mutagen in this system) was tested as a positive control. In addition to the study of the growth inhibitory activity of this chemical at a selected concentration, the full mutation assay was performed. This assay included: a determination of fold growth over the post-treatment expression period of greater than 60 hr; a cloning efficiency study in soft agar of the cells present after the expression period to determine their reproductive integrity (survival); and a selection procedure in soft agar using trifluorothymidine (TFT) to detect any cells which had been forward mutated by the EMS treatment. These cells would lack the enzyme thymidine kinase, and therefore would be TFT resistant.

B. METHODS

1. Treatment

This experiment was performed by using the partial medium replacement method described above for cell distribution. A stock concentration of EMS, prepared within 1 hr of treatment by pipetting 0.171 ml of EMS into 1.83 ml of non-sterile saline, was sterile-filtered through a 0.22 μ millipore filter unit. For treatment, 0.12 ml of this filtered solution was pipetted into replicate T-25 flasks containing 600,000 cells/ml in 11.88 ml of medium. The washing procedures after treatment were performed as already described.

2. Cell Distribution for Fold-Growth and Growth Curve Studies

Prior to the final cell count, 5 ml of the final 10-ml washed cell suspension was distributed into 50-ml sterile rubber-stoppered glass Erlenmeyer flasks. These flasks had previously been loaded with 5 ml of fresh warm F_{10p} medium (no HEPES), and gassed for 1 sec with 5% carbon dioxide and 95% air. The flasks were placed in a box (to minimize light exposure) and then placed on the platform in a New Brunswick rotating incubator at 37°C. They were designated: "fold-growth" flasks.

3. Growth Curve Study

The cell density in the remaining 5 ml of washed cell suspension was then determined by Coulter counter; and the growth curve study in 60 ml of F_{10p} medium in 250-ml Erlenmeyer flasks was initiated as previously described.

4. Fold-Growth and Soft Agar Procedures

On each morning after the day of treatment (day 0) -- i.e., on day 1 (approximately 16-hr elapsed time), and on day 2 (approximately 40-hr elapsed time)--a cell count (Coulter count) was performed on each of the fold-growth flasks (2 x 0.5 ml aliquots). That volume in the flask containing 3×10^6 cells was determined and retained in the flask; any extra cell suspension was discarded. The volume of each flask was then adjusted back to 10 ml with fresh warm F_{10p} medium, and the flasks were reincubated. The flasks were not regassed on days 1 and 2.

On day 3, 250-ml flasks containing 0.4% cloning medium (CM) were prepared. For each treatment flask, a set of three 250-ml flasks was prepared and gassed: One flask contained 100 ml of CM, and was labeled 'TFT', another contained 50 ml of CM, and was labeled '1:50'; and a third contained 100 ml of CM and was labeled 'VC'. These flasks were returned (after preparation) to the rotatory incubator, which was set at 100 rpm and 39°C. At an elapsed time of approximately 65 hr, a final Coulter count was performed on the fold-growth flasks. The volume in each fold-growth flask containing 3×10^6 cells was determined. These volumes were transferred into sterile 15-ml Corning tubes, and centrifuged as previously described to pellet the cells. All but 1 ml of medium was discarded from each tube.

The standard soft agar cloning-mutant selection procedure was then followed. The cells were first resuspended (one treatment flask tube at a time) in the 1 ml of remaining medium with a sterile pasteur pipet by repipetting up and down 5 times. Using the same pasteur pipet, the 1 ml was transferred into the 250-ml flask labeled 'TFT'. The TFT flasks were removed from and returned to the rotating incubator one at a time. After these transfers had been completed (resulting in cell densities of up to 30,000 cells/ml), and working again with one treatment condition at a time, a 1-ml aliquot of cell suspension was transferred from the TFT flask into the flask marked 1:50. This dilution gave a cell density of 600 cells/ml. A 1-ml volume of sterile trifluorothymidine (TFT) (Lot No. 81F-00811, Sigma Chemical Co., St. Louis, Mo.) at 100 µg/ml was then added by 1-ml pipet into the TFT flask (the pipet was rinsed with the CM in the flask). Both flasks were swirled by hand and returned to the rotating incubator. After this set of transfers had been completed, and again working with one treatment condition at a time, a 1 ml aliquot of cell suspension was transferred from the 1:50 flask into the VC flask. This dilution resulted in a cell density of 6 cells/ml. The 1:50 flask was discarded; the VC flask was returned to the rotating incubator.

The next step was the distribution of 33-ml aliquots of the cell suspension in CM in the TFT flasks into 20 x 100 cm petri dishes. The three dishes for each TFT flask were kept on a warming plate in the hood during this procedure. The contents of one 250-ml TFT flask were distributed into the three petri dishes. After this distribution, the dishes were placed in the freezer compartment of the refrigerator (on a metal rack with no stacking of dishes) for 12 min.

Meanwhile, the next TFT flask contents were being distributed into three dishes. After 12 min in the freezer, the three dishes of the first TFT flask were stacked and placed inside the laminar flow hood to warm them towards room temperature. They stood inside the hood for 5 min, and were then transferred into the 37°C incubator. After all of the TFT flasks were likewise distributed, the process was repeated for the VC flasks. The dishes were then incubated for 11 days, at which time the colonies were counted and sized on an automatic Colony Counter (Artek Systems Corp., Farmingdale, N.Y.).

C. RESULTS

The results of this experiment are shown in Fig. 30 and Table 2. The treatment with EMS at 1 mg/ml is clearly growth-inhibitory. The data plotted are the average of values for 4 growth curves from 2 independent treatment flasks.

As shown in Table 2, the average cloning efficiency in the viability assay for the control cells was 25% (100% survival), while the average cloning efficiency for the cells treated with EMS was 12.7%. The percent survival of the cells treated with 1 mg/ml EMS and still present in the cell suspension at the time they were suspended in soft agar (65.5-hr expression time) was, therefore, 50.8% (using the 25% C.E. of the Control as 100% survival).

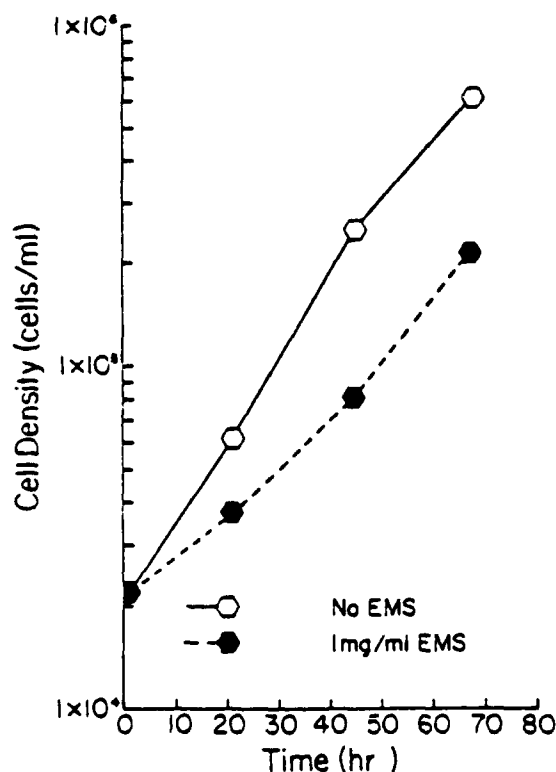


Figure 30. Growth curve of L5178Y cells after a 4-hr incubation at 37°C with 1 mg/ml of ethylmethanesulfonate (EMS). The data are averaged for four growth curve flasks initiated from two individual treatment flasks.

TABLE 2. EMS MUTATION EXPERIMENT

CHEMICAL & CONC. μg/ml	ACTUAL CELL CONC. (X 10 ⁵)	ACTUAL VOLUME OF MEDIUM ml	ACTUAL CELLS CENTRIFUGED (X 10 ⁶)	TFT CELL CONC. (X 10 ⁴ /ml)	TFT CELLS in 33 ml (X 10 ⁴)	VC 1/5000 TFT CELL CONC. cells/ml
EMS, 0 μg/ml A	7.25	4.14	3.0	3.0	99.0	6
0 μg/ml B	6.25	4.80	3.0	3.0	99.0	6
1000 μg/ml A	5.31	5.65	3.0	3.0	99.0	6
1000 μg/ml B	5.52	5.43	3.0	3.0	99.0	6
CHEMICAL & CONC. μg/ml	VC CELLS in 33 ml	TOTAL VC COLONIES per DISH	PERCENT CE (%)	PERCENT SURVIVAL	TOTAL TFT COLONIES per DISH	MUTATION FREQUENCY per 10 ⁶ VC
EMS, 0 μg/ml A	198	57,64,68 Avg = 63	28.8, 32.3, 34.3 Avg = 31.8	100	89,87,86 Avg = 87	282.7, 276.4, 273.2 Avg = 277
0 μg/ml B	198	31,30,48 Avg = 36	15.7, 15.1, 24.2 Avg = 18.3	Avg CE = 25.05	66,69,55 Avg = 63	364.2, 380.8, 303.5 Avg = 350
1000 μg/ml A	198	19,23,23 Avg = 22	9.6, 11.6, 11.6 Avg = 10.9	38.3, 46.3, 46.3 Avg = 44	191, 230, 183 Avg = 201	1770.2, 2131.6, 1696.0 Avg = 1866
1000 μg/ml B	198	37,23,26 Avg = 29	18.7, 11.6, 13.1 Avg = 14.5	74.7, 46.3, 52.3 Avg = 58	225, 177, 216 Avg = 206	1566.9, 1232.6, 1504.1 Avg = 1434

A,B = independent treatment flasks

CE = cloning efficiency

CONC. = concentration

EMS = ethylmethanesulfonate

TFT = trifluorothymidine

VC = viable cells

The spontaneous mutant frequencies per 10^6 viable cells for the two independent control flasks were 277 and 350, respectively; the average was 314. The mutant frequencies per 10^6 viable cells for the two independent EMS treatment flasks were 1,866 and 1,435, respectively; the average was 1,651. The induced mutant frequency (treated minus control) was therefore 1,337 mutants per 10^6 viable cells.

XII. EFFECT OF STATIONARY VS. AGITATED MOTION ON SUSPENSION GROWTH

A. OBJECTIVE

Preliminary experiments were undertaken to evaluate the growth characteristics when L5178Y cells were grown in 15-ml conical tubes which remained stationary during the greater than 50 hr of the growth curve experiment, and in 15-ml tubes which were maintained in constant motion, either on a rocker platform or in a rotatory incubator.

B. METHODS

For comparison of these two growth techniques, cells were mass cultured in a volume of 60 ml in a 250-ml flask so that 8.5×10^6 cells were available. After an initial cell count, a volume containing 8.5×10^6 cells was adjusted to a final volume of 170 ml with fresh warm F10p medium. This resulted in a cell density of 50,000 cells/ml. Equal 5-ml aliquots of the cell suspension were then distributed into thirty Corning sterile 15-ml centrifuge tubes. The tubes were placed on their side in two racks, and 15 tubes (in each rack) were incubated either stationary in the 37°C air incubator or on a rocker platform for more than 30 hr, followed by rotation for the remainder of the experiment (beginning at 48 hr). Immediately after distribution (and at later times), 2 tubes were randomly picked from the rack for cell counting. Trypsin in saline served as the background count.

C. RESULTS

As can be seen in Fig. 31, continuous movement of the tubes resulted in a higher maximum cell density, approaching 1×10^6 cells/ml. The stationary tube system resulted in a maximal cell density of under 6×10^5 cells/ml; this system is not preferred.

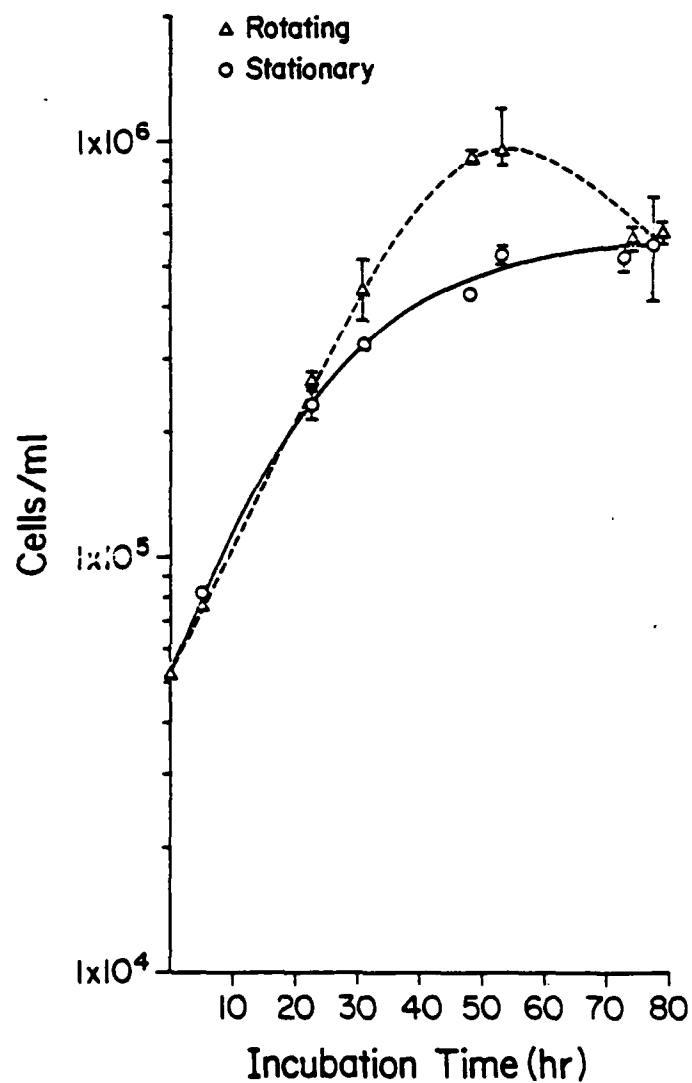


Figure 31. Growth curves of L5178Y cells (untreated) upon incubation, either stationary or with constant agitation (rocking and rotation). The data are the average of two tubes per point.

XIII. COMPARISON OF L5178Y CELL GROWTH IN A 5% CO₂ GAS ATMOSPHERE VS. IN AIR USING HEPES-CONTAINING MEDIUM (10 mM or 15 mM)

A. OBJECTIVE

The purpose of this experiment was to compare the growth of L5178Y cells when carbon dioxide in air was used to adjust the pH of the medium versus that when HEPES buffer was used to control the pH.

B. METHOD

Three 250-ml sterile Corning Erlenmeyer screw-cap flasks, containing a final volume of 60 ml of medium, were first prepared and warmed to 37°C. One flask contained 60 ml of F_{10p} medium and was gassed with 5% carbon dioxide and 95% air to adjust the pH. The second flask contained F_{10p} medium with 10 mM HEPES final; it was not gassed. The third flask contained F_{10p} medium with 15 mM HEPES final; it also was not gassed.

The cells used had previously been purified of spontaneous mutants by the standard THMG-THG treatment sequence. The density of the cells in a mass culture flask was determined, and 1.2×10^6 cells were distributed into three 15-ml centrifuge tubes and pelleted at 800 rpm (129 x g) for 8 min. The supernatant was discarded. The cells were then resuspended in an aliquot of medium transferred from each of the three flasks just described; the concentrated cell suspension was then returned to the appropriate flask. The final cell density was approximately 20,000 cells/ml.

The three flasks were returned to the rotatory device in the 37°C air incubator. After a few minutes of incubation, to allow for equilibration, two 1.0-ml aliquots were removed from each flask to obtain an accurate initial cell count (by Coulter counter). A 5-ml volume of cell suspension was also removed for a pH determination using an Orion pH meter.

Cell counts, or cell counts and pH determinations, were subsequently performed at daily intervals.

C. RESULTS

The results, presented in Fig. 32, show equivalent growth patterns; i.e., similar increases in cell density with incubation time. The pH change in the 5% carbon dioxide and 95% air flask was to a slightly more acidic value than that in the HEPES buffered flask; this difference was so slight that the buffering systems appeared to be of equivalent usefulness for future experiments. If HEPES were to be employed, the 10 mM concentration would be used.

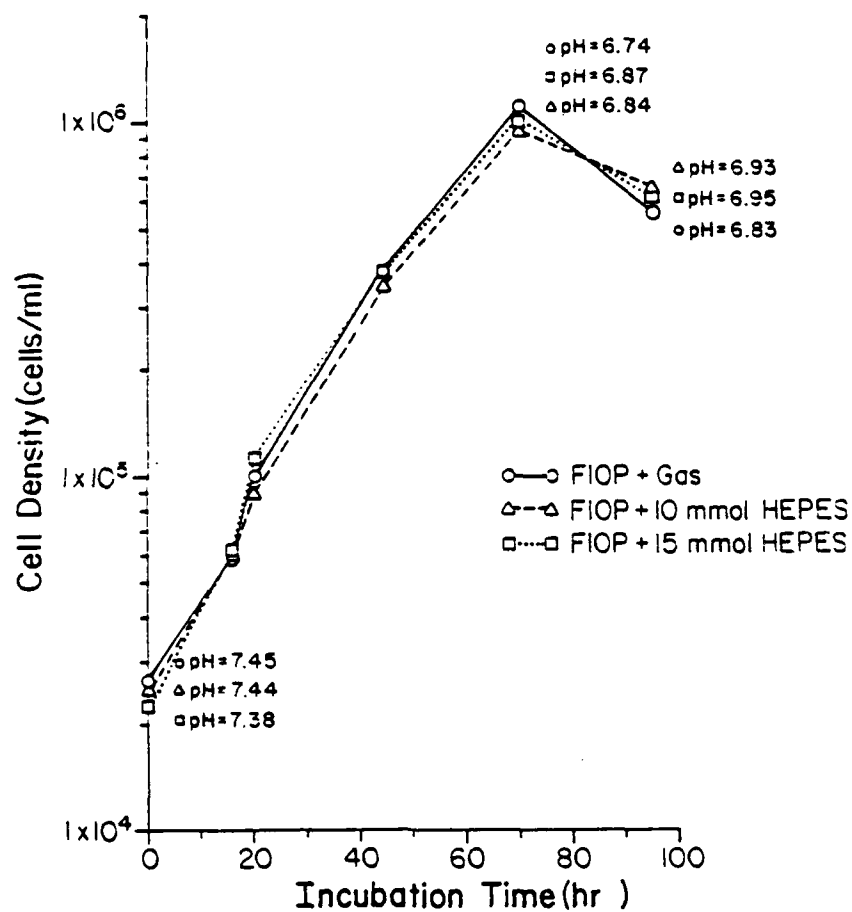


Figure 32. Growth curves of L5178Y cells (untreated) in medium with different buffering systems. Incubation was in 250-ml Erlenmeyer flasks with continuous rotation at 37°C.

XIV. REFERENCES

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